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The STAT3-IL10-IL6 pathway is a novel regulator of macrophage efferocytosis and phenotypic conversion in sterile liver injury

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Abstract

The disposal of apoptotic bodies by professional phagocytes is crucial to effective inflammation resolution. Our ability to improve the disposal of apoptotic bodies by professional phagocytes is impaired by a limited understanding of the molecular mechanisms that regulate the engulfment and digestion of the efferocytic cargo. Macrophages are professional phagocytes necessary for liver inflammation, fibrosis and resolution, switching their phenotype from pro-inflammatory to restorative. Using sterile liver injury models, we show that the STAT3-IL10-IL6 axis is a positive

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Author contributions

LC designed and performed the experiments, analyzed the data and wrote the manuscript. PSL performed experiments, helped in the design of the *in vitro* phagocytosis using live imaging, helped with the writing of the materials and methods and with the revision of the manuscript. AP helped designing the preliminary experiments, performed experiments and helped with the set-up of the image analysis for immunohistochemistry. SFG, EL, RLA, SEM, KLH performed experiments. SNG helped with the animal work. TJK acquired pictures with the multi-slide scanner from all the H&E sections and wrote the macro to analyze the necrotic area in APAP dosed mice. EOD gave crucial support with the technical development and the analysis of the *in vitro* live imaging experiments. DV helped with the design of the experiment for NF- κ B role in phagocytosis and performed experiments. YF helped in the design of the experiments with zebrafish, helped in performing experiments, provided full materials and methods section for the zebrafish experiments and revised the manuscript. LB helped in designing and performing gene analysis experiments and revised the manuscript. CG and NCH helped in designing experiments and revised the manuscript. SMA, wrote the project, helped defining the experimental strategy and provided support in data interpretation. SJF and JPI wrote the project, helped defining the experimental strategy, provided support in data analysis and interpretation, and contributed to manuscript preparation, editing and revision.

regulator of macrophage efferocytosis, survival and phenotypic conversion, directly linking debris engulfment to tissue repair.

Keywords

macrophages; efferocytosis; liver; sterile injury; IL6; IL10; STAT3; macrophage polarization

Introduction

Phagocytosis is an evolutionarily conserved, multi-step process that spans the recognition and engulfment of phagocytic cargo, through cargo processing to antigen presentation (1). Prompt removal of apoptotic and necrotic cells (efferocytosis) is critical to immune tolerance induction and maintenance or re-establishment of tissue homeostasis (2). A failure of efficient efferocytosis has been implicated in the pathogenesis of autoimmune and inflammatory disorders (3–5) such as Systemic Lupus Erythematosus (SLE). Chronic inflammatory conditions are characterized by an aberrant release of pro-inflammatory intracellular components from secondary apoptotic and necrotic cells that fail to be cleared (6, 7). In solid tumors, recognition of apoptotic cells can promote an immunogenic response and anti-tumoral acquired immune responses (8). Efferocytosis has been postulated to be a mechanism through which initial organ damage, i.e. cell death, programs tissue remodeling and regeneration. However, the molecular mechanisms underpinning this are not well understood (1, 9). Understanding the mechanisms underpinning this process could allow therapeutic targeting. Macrophages are professional phagocytes (10), and they change phenotype depending on micro-environmental cues which include efferocytosis (11–13). We and others have previously characterized a hepatic restorative macrophage, derived from recruited inflammatory macrophages that is necessary for tissue remodeling and regeneration following sterile liver injury (14, 15). Furthermore in liver injury, phagocytosis of necrotic hepatocytes by macrophages prompts Wnt ligand secretion which promotes liver regeneration (16).

In this study, we have derived macrophages from the bone marrow of control (Gpnmb⁺) mice or of mice that have a defect in Glycoprotein Non-Metastatic Melanoma B (Gpnmb⁻), an interactor of Light Chain 3 (LC3). Gpnmb⁻ macrophages will engulf but fail to process their apoptotic cell cargo (17, 18). Macrophages have been fed with apoptotic thymocytes to study the activation of intracellular pathways downstream from the engulfment and digestion of phagocytic cargo. We show that macrophage phosphoSTAT3 (pSTAT3) is a pro-phagocytic mediator that enhances macrophage phagocytosis, via rapid non-transcriptional regulation of IL10. pSTAT3 activation is sustained at the later stages of phagocytosis, promoting IL6 transcription and thereby making efferocytosis more efficient. Following acute and chronic sterile liver injury, Gpnmb⁻ mice exhibited extensive liver damage and increased numbers of pro-inflammatory macrophages. Treatment with recombinant IL6 is sufficient to increase efferocytosis *in vivo* and to promote macrophage phenotypic conversion to pro-restorative macrophages.

Materials and Methods

Mice

C57BL/6 mice (CD45.2+) were purchased from Charles River UK. C57BL/6 IL6-KO mice (28) were provided by Prof S. Anderton, University of Edinburgh. Gpnmb+ (DBA/2J-Gpnmb+/SjJ) and Gpnmb- (DBA2J) mice were originally imported from Jackson Laboratories (19). The colony was propagated at the University of Edinburgh. Mice were housed in groups of five/six in open-top cages and synchronized to a 10-14-hour dark/light cycle with access to food and water ad libitum. Mice were bred under specific pathogen-free conditions at the University of Edinburgh. All experiments had local ethical approval and were conducted under UK Home Office Legislation. Genotyping was carried out by using PCR by TransnetYX.

Liver Fibrosis Model

Wild-type C57BL6 male mice were allowed to acclimatize for a minimum of one week in a clean animal facility. Prior to Carbon Tetrachloride (CCl₄) treatment, mice were randomly assigned to treatment groups. Adult male mice (10-12 weeks old) were used. Hepatic fibrosis was induced by two injections per week of CCl₄ (0.4 µL/g; Sigma) i.p., diluted 1:3 in olive oil (Sigma) for 6 weeks. Animals were culled at stated time points after the final CCl₄ injection.

APAP-induced liver injury and BMDM administration

Wild-type C57BL6 male mice (10 weeks old) were allowed to acclimatize for a minimum of one week in a clean animal facility. Prior to acetaminophen (APAP) administration, mice were fasted at least 12 hours (h). Mice received a single injection (i.p.) of APAP (300 mg/kg) dissolved in warm saline between 23:00 and midnight. Mice were left to recover until morning on a heated mat or in a warm rack; at indicated time points, they were humanely culled according to local ethical guidelines.

Acute liver damage by single injection of CCl₄

Adult male mice at least 8 weeks old were used. Acute liver injury was induced by i.p. injection of CCl₄ (0.4 µL/g; Sigma) diluted 1:3 in olive oil (Sigma). Animals were culled at stated time points after the CCl₄ injection.

Phagocytosis Assay *in vivo*

Mice dosed with APAP or receiving a single CCl₄ injection were injected with 100µL 0.1mM PKH26PCL diluted 1:10 according to manufacturer's instructions (Sigma Aldrich) i.v. via tail vein to label phagocytic cells. Control mice were injected with 100uL of diluent only. Percentage (%) of phagocytic cells in hepatic infiltrating and resident is reported. Mice were culled 6h post-CCl₄ and livers were harvested and process as described for flow cytometry analysis. Numbers were calculated based on the cell counting performed after isolation of the non-parenchymal cell fraction. The percentage of phagocytic cells was calculated on the gate of viable CD45+Ly6G-CD3-CD19-NK1.1- cells

BMDM adoptive transfer

We transferred Gpnmb+ CFSE-labelled BMDMs and Gpnmb- CMTMR-labelled BMDMs into C57Bl/6 mice receiving a single dose of CCl₄. Mice received either Gpnmb+ or Gpnmb- BMDMs or both population in a 1:1 ratio. To avoid an effect of the labelling on the parameter analysed the experiment was repeated using Gpnmb+ CMTMR-labelled BMDMs and Gpnmb- CFSE-labelled BMDMs. CFSE-labelled or CMTMR-labelled BMDMs were resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS) and administered (2.5×10^6 cells, 100 μ L) via tail vein to mice under gaseous isoflurane/oxygen anaesthesia at 2h after the single CCl₄ injection. Mice were humanely culled by asphyxiation in a rising CO₂ atmosphere. Death was confirmed by neck dislocation. CFSE-labelled or CMTMR-labelled BMDMs were injected 2h after CCl₄ dosing. CFSE and CMTMR labelling efficiency was routinely >90%. Viability post transfer was routinely >90%. Mice were culled 6h post-CCl₄ and livers were harvested and process as described for flow cytometry analysis.

Transferred BMDMs were identified as CFSE+ or CMTMR+. The percentage of CFSE+/CMTMR+ cells was calculated on the gate of total viable CD45+Ly6G-CD3-CD19-NK1.1- cells. The negative was set on a liver from a non-transplanted mouse. The percentage of Ly6C+ and Ly6C- was calculated on the gate of CFSE+/CMTMR+ cells. The negative was set using the liver from a non-transplanted animal (for single CFSE+ and CMTMR+) or transplanted with BMDMs labelled with the other tracker (CFSE for CMTMR and vice versa).

Samples isolation and storage

Whole blood was collected via cardiac puncture or from the inferior vena cava using 30uL heparin (200U/mL)/samples. Blood was centrifuged at 10000 rpm for 10' at room temperature in a bench centrifuge (Eppendorf) and plasma was isolated and snap frozen using dry ice. Plasma was then stored at -80 °C and used for protein dosages. Liver tissue was harvested and the left lateral lobe was separated into two pieces and placed in either a freezing isopentane bath or fixed in methacarn for 24h. The remaining liver and other organs were fixed in formalin (4% paraformaldehyde) for 24h before paraffin-embedding. The central lobe was used for isolation of hepatic non-parenchymal cells and flow cytometry analysis.

Isolation of hepatic non-parenchymal cells

Isolation of hepatic non-parenchymal cell (NPC) fraction was performed as described in (14) (20) with minor modifications. Briefly, mouse livers in situ were perfused with 5 mL 0.9% NaCl solution through the inferior vena cava followed by cutting of the portal vein to remove circulating cells. Livers were then harvested and weighed; the right lobe was homogenized using a scalpel and digested in RPMI 1640 containing Collagenase V (Sigma, 0.8mg/ml), Collagenase D (Roche, 0.625mg/ml), Dispase (Gibco, 1mg/ml), Collagenase D (1.6 mg/mL; Roche) and DNase I (100 μ g/mL; Roche) for 25 min at 37 °C, shaking vigorously every 5 min. Digested livers were passed through 70 μ m cell strainers, and enzymes were inactivated by the addition of RPMI 1640 with 10% FCS. The non-parenchymal cell fraction containing hepatic macrophages was harvested by two centrifugations at 300 $\times$ g, 4 °C, 5', followed by red cell lysis with 3mL 1x lysis buffer (BD

Pharm Lyse™, BD Bioscience) for 5 min on ice. Cells were then counted, and used for flow cytometry.

Non-parenchymal cell labelling and flow cytometry analysis

Nonspecific antibody binding was blocked by incubating cells with 10% mouse serum for 20' at 4 °C followed by incubation with combinations of primary antibodies (each used at 1/200 dilution) for 20' at 4 °C. The following conjugated antibodies were used: CD11b BV650 (clone M1/70; Ebioscience), Ly-6C V450 (clone HK1.4; Ebioscience), CD45.2 AF700 (clone 104; Ebioscience), F4/80 APC (dilution 1:100; clone BM8; Invitrogen), Ly-6G PE-Cy7 (clone 1A8; Biolegend), CD3 PE-Cy7 (clone 17A2; Biolegend), NK1.1 PE-Cy7 (clone PK136; Biolegend), CD19 PE-Cy7 (clone 6D5; Biolegend). Cell viability was assessed with Fixable Viability Dye eFluor780 (1:1000, Ebioscience) according to manufacturers' protocols. After antibody staining, samples were either analyzed immediately or fixed with 10% buffered formalin. Data were analyzed using FlowJo10 software (Treestar Inc., US). Hepatic RESIDENT macrophages were defined as viable CD45+Ly-6G-CD3-NK1.1-CD19-CD11b^{low}F4/80^{high}. Hepatic infiltrating macrophages were defined as: viable, CD45+Ly6G-CD3-NK1.1-CD19-CD11b^{high}F4/80^{low} cells from NPC fraction of digested livers and used to identify macrophage subsets. Subsets were expressed as proportions of total hepatic macrophages or CD45+cells. Quantification of absolute numbers of cells per liver was performed by expressing each subset as a proportion of NPCs, counting total number of NPCs in the digested portion of liver, calculating the total number of NPCs in the whole liver by weight differential, and thus, calculating the total number of each subset. Circulating monocytes (from whole blood diluted 1:1 in heparin) were stained using BD Pharm Lyse™ (BD Bioscience) before analysis. Monocytes were identified as CD45+CD11B+Ly-6G-CD3-Ly-6C^{high} and Ly-6C^{low} cells from whole blood and expressed as a percentage of total peripheral mononuclear cells.

Plasma chemistry evaluation

Plasma chemistry was performed by measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and plasma albumin. ALT was measured using the method described in (21), utilising a commercial kit (Alpha Laboratories Ltd). AST and ALP were determined by a commercial kit (Randox Laboratories). Total bilirubin was determined by the acid diazo method described by Pearlman and Lee (22) using a commercial kit (Alpha Laboratories Ltd). Mouse plasma albumin measurements were determined using a commercial serum albumin kit (Alpha Laboratories Ltd). All kits were adapted for use on a Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd). For all assays, intra-run precision was CV < 4%.

Phagocytosis Assay *in vitro* – flow cytometry

Bone marrow-derived macrophages (BMDMs) were prepared as described (Henderson NC, et al. 2008) (23) from adult male C57BL/6 mice. Briefly, we differentiated whole bone marrow for 7 days at 37 °C, 5% CO2 in DMEM/F12+Glutamax (Gibco) medium with 10% FCS and 25µg/mL recombinant murine M-CSF (Miltenyi Biotec) under non-adherent conditions using Ultra Low Attachment Flasks (Corning). This process routinely yielded a macrophage population of >90% purity as assessed by flow cytometry for CD11b. Apoptotic

thymocytes were prepared as previously described (24). Briefly, thymuses were removed from C57BL/6 mice, ages 3–5 wks, homogenized in RPMI medium, and incubated with hydrocortisone (1 μ M; Sigma Aldrich UK) and 1% FCS at 37 °C, 5% CO₂ for 16 h. This process routinely yielded a population of dead thymocytes with over 80% trypan blue-positive. Apoptotic thymocytes were labelled using CMTMR (Invitrogen) as described in (25). Briefly, macrophages were challenged with apoptotic cells for 7 min, 15 min, 30 min, 1h and 2 h at a 1:5 ratio at 37°C or 4°C. Cells were washed and phagocytosis verified by flow cytometry after staining with anti-CD11b BV650 (clone M1/70; Ebioscience) and anti-Ly-6C V450 (clone HK1.4; Ebioscience). When the assay was performed in a 96-well plate (Ultra-low attachment, Corning Costar) cytochalasin D 10 μ M (Sigma-Aldrich) was used as a negative control. The phagocytosis was calculated as percentage of CD11b+CMTMR+ cells at 37°C – %CD11b+CMTMR+ cells at 4°C. Percentage of Ly6C+ cells was calculated in the gate of CD11b+CMTMR+ cells at 37°C. Mean fluorescence intensity for CMTMR was calculated on the same gate in the same conditions. Data were acquired on a LSRII Fortessa (BD Biosciences) when the assay was performed in tubes. Data were acquired and analyzed on a NovoCyte 3000™ (Acea Biosciences, USA) when the assay was performed on a 96 well-plate.

Phagocytosis Assay *in vitro* – Live imaging (Operetta™, Perkin Elmer)

BMDMs were plated (1x10⁵/well) in 96-well CellCarrier microplates (PerkinElmer) overnight before stimulation with appropriate cytokines or blockers (see methods below). Before imaging, BMDMs were stained with NucBlue live cell stain (ThermoFisher) and CellMask Deep Red (ThermoFisher) plasma membrane stain according to the manufacturer's instructions. Plates were transferred to Operetta high-content imaging system (PerkinElmer) and allowed to equilibrate at 37 °C and 5 % CO₂. Phagocytosis was initiated by the addition of pHrodo green zymosan bioparticles (ThermoFisher) to the wells. Fluorescent images were taken in the DAPI channel, 488 nm, and 647 nm before, and at 5 min intervals after the addition of bioparticles for a maximum of 150 mins. Images were quantified on Columbus image analysis software (PerkinElmer). Macrophages positive for phagocytosis were classified based on a fluorescence intensity (488 nm) greater than 500 and expressed as a fraction of all live cells (NucBlue positive cells). Mean fraction values were taken from four separate wells per group.

Study of the pSTAT3-IL10-IL6 pathway

The role of pSTAT3-IL10-IL6 pathways in phagocytosis was investigated *in vitro* in the presence or absence of recombinant murine (rm)-IL6 (Miltenyi Biotec) at distinct concentrations: 1, 5 and 50 μ g/mL. A pSTAT3 (Tyr705) inhibitory peptide or an irrelevant peptide (MERK Millipore) were used at a 30 μ M final concentration *in vitro*. rmIL10 was used at a final concentration of 10ng/mL (Miltenyi Biotec) (25). For the Operetta™ live imaging experiment BMDMs were treated with pSTAT3 inhibitory peptide either 2h before the phagocytosis or during the phagocytosis itself.

For immunofluorescence (IF) of pSTAT3, BMDMs were fixed at distinct time points after the start of phagocytosis using PFA 4% for 15 min. Permeabilization was performed using TritonX100 (Sigma-Aldrich) for 15 min. To minimize non-specific binding, we added

protein block solution (SpringBio) for 30 min. Primary antibodies used were: Phospho-Stat3 (Tyr705) (clone D3A7) XP® Rabbit mAb #9145 1µg/mL (Cell Signalling Technologies); GPNMB (K-16) Goat pAb #Sc-47006 1µg/mL (Santa-Cruz). Primary antibodies were incubated 3h, RT. Secondary antibodies were AlexaFluor488 and AlexaFluor555 respectively. Secondary antibodies were incubated in the dark for 1h at RT. BMDMs were then mounted on a slide using an aqueous mounting medium (Fluoromount-G®, SoutherBiotech) and imaged using a Leica SP5 confocal microscope. Alexafluor 488 and 555 were detected using band paths of 495–540 and 561–682 nm for 488 and 543 nm lasers respectively.

The role of pSTAT3-IL6 pathways in phagocytosis was investigated *in vivo* injecting 50 µg/mouse of rmIL6 (Miltenyi Biotec) i.p. IL6 was injected together CCl₄ in the single CCl₄ acute liver damage model. IL6 was injected either together or 2h after the administration of APAP. In the chronic model of CCl₄ intoxication IL6 is injected at the same time of CCl₄ suspension to investigate the effect on the macrophage phenotype in Gpnmb⁺ and Gpnmb⁻ mice. A STAT3 inhibitory peptide or an irrelevant peptide (MERK Millipore) were used at a 30 µM final concentration following the same treatment regimen in the acute damage model by single CCl₄ injection and in the model of APAP intoxication.

RNA isolation and qPCR

For whole liver, the caudate lobe was snap frozen in liquid nitrogen and stored at -80 °C. RNA was extracted using QIAshredder columns and RNeasy mini columns (Qiagen) according to the manufacturer's protocol, followed by quantification using the Nanodrop Spectrophotometer (Thermo Scientific); 0.5µg RNA was reverse-transcribed using SuperScript III (Invitrogen) according to the manufacturer's protocol. Gene expression was calculated using the $\Delta\Delta CT$ method relative to housekeeping gene β -actin and Gapdh. For the *in vitro* phagocytosis assay, RNA was extracted using the QIAshredder columns and RNeasy mini columns (Qiagen), and 100 ng RNA were reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. cDNA was then diluted to 1:10 with RNase Free water, prior to qPCR analysis. The following QuantiTect Primer Assays (Qiagen) were purchased: Tgf- β , Il10, Il10, Il6, Hmgb1, Sdf1/Cxcl12, Mcp1/Ccl2, Socs3, col1a2 and col3a1, Mmp2, Mmp7, α -SMA. Genes were analysed using the Quantifast SYBR Green PCR Kit (Qiagen) on an ABI 7500 Fast Real-Time System or a Roche LightCycler480 according to the manufacturer's instructions.

Immunohistochemistry

Liver tissue was harvested and fixed overnight in 10% neutral buffered formalin or Methacarn solution followed by paraffin embedding. Tissue sections were deparaffinised with xylene and rehydrated using alcohol (100%, 75% and 65%). They were then subjected to antigen retrieval by pre-treating in a microwave oven with TRIS EDTA pH9 for CD3 and Ki67 antigen detection. Antigen retrieval for α -SMA, Collagen 1 and 3 was performed by pre-heating sections in a microwave oven with citric acid. The following primary antibodies and conditions were used: CD3 (rabbit polyclonal, AbCam), Ki67 (rabbit polyclonal, Dako, discontinued), collagen 1 (131008; 1/100 dilution; formalin-fixed; antigen retrieval; Southern Biotech, collagen 3 (131001; 1/100 dilution; formalin-fixed; antigen

retrieval; Southern Biotech), α -smooth muscle actin (α -SMA; clone 1A4; 1/4,000 dilution; formalin-fixed; antigen retrieval; Sigma-Aldrich). Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide, and protein block solution (Dako). To minimize non-specific binding, Normal Goat Serum was added. Appropriate biotinylated secondary antibodies were used. Immunostaining was developed using 3,3'-diaminobenzidine, DAB (Dako), and counterstaining with Harris's Haematoxylin. Positive cells/area were measured from 20 random fields at x20 magnification. Sections were photographed using a Nikon Eclipse E600 microscope and NIS-Elements D3.1 Software. Hematoxylin and eosin staining (H&E) was performed according to standard protocols. Morphometric pixel analysis to quantify histological staining was performed. For necrosis quantification, H&E stained section were scanned to create a single image with Dotslide VS-ASW software (Olympus) using a motorized stage and an Olympus BX51 microscope, acquiring images using an Olympus PlanApo 2X lens and Olympus XC10 camera. Images were analyzed using the Trainable WEKA Segmentation plugin in FIJI. A separate classifier identifying necrotic and viable tissue was determined and applied to all tissue in each image.

Human monocyte derived macrophages

Human monocyte derived macrophages (hMDMs) were differentiated from cryopreserved CD14 monocytes essentially as described previously (26). Briefly, cryopreserved stocks were thawed rapidly and diluted in Iscove's Modified Eagle's Medium (IMEM) supplemented with 10 % FBS (v/v), 2 mM glutamine, penicillin/streptomycin (500 U/mL, 500 μ g/mL), and 100 ng/mL human recombinant CSF1 (Miltenyi) at 2×10^6 cells/mL in ultra-low attachment flasks (Corning). Cells were differentiated towards macrophages for 7 days with a 10 % media change every second day containing 1 μ g CSF1. After 7 days, hMDMs were harvested, counted, and plated on CellCarrier plates for *in vitro* phagocytosis assays using the Operetta instrument for live imaging (see above).

Zebrafish strain & maintenance

Adult zebrafish (*Danio rerio*) strain TgBAC(csf1ra:GAL4-VP16;UAS:mCherry (27) were maintained at 28°C and crossed as previously described (28). Embryos were maintained at 28°C and staged according to standard protocols (28).

Zebrafish larval tail fin regeneration assays

Sterile tail fin amputation was performed as previously described (29). Briefly, zebrafish embryos at 2 days post-fertilization were anesthetized in 0.3% Danieau's solution containing 0.1 mg/ml Tricaine (ethyl 3-aminobenzoate, Sigma) and tail fin were cut off from the end of notochord using a scalpel. Regeneration was monitored at 24h, 48h and 72h after wounding. Photograph of the regenerating fin were taken at 40x or 50x using a Leica M205 stereomicroscope.

Treatment with small molecules

5,15-DPP (5,15-Diphenyl-21H,23H-porphine, 5,15-Diphenylporphyrin, 5,15-diphenyl-Porphine) was dissolved in DMSO (stock 10 mM) and diluted to a concentration of 400 μ M and 2mM in 0.3% Daneau's solution. Larvae were treated with compounds at their final

concentration immediate after amputation, medium were changed daily with fresh 0.3% Daneau's solution containing the compound.

Statistics

All data are expressed as mean $\pm$ standard deviation (SD). The number of replicates is indicated in each figure and each replicate represents a biological rather than an experimental replicate. Data are analysed and graphs are generated with GraphPad Prism version 5 or 6 (GraphPad Software, Inc, USA). Statistic tests have been chosen depending on the biological question behind the experiment. Briefly, we use one- or two- way ANOVA followed by an appropriate post-hoc test. The test used is stated in each figure legend. $p < 0.05$ is considered statistically significant.

We have performed a power calculation for the number of mice to use in the studies on the chronic CCl₄ model based on a pilot study on Gpnmb⁺ and Gpnmb⁻ mice on the ALT measure (indicating liver damage) at 48h after the withdrawal of CCl₄. We have assumed a μ_1 of 150 for Gpnmb⁺ and a μ_2 of 600 for Gpnmb⁻, with a sigma of 150. We have set the power desired at 0.80 assuming a statistical significance at the threshold of 0.05. The power calculation returned an $n=3$. This is the minimal number of mice used in each experiment.

For the *in vivo* phagocytosis there were no preliminary data available. We have treated from a minimum of 3 to a maximum of 7 mice/group. The parameter analysed for the power calculation is the %phagocytic infiltrating inflammatory macrophages. If we assume a μ_1 (Gpnmb⁺) of 80 and a μ_2 (Gpnmb⁻) of 20 as derived from our experiment and a sigma of 15, the power calculation for a statistical significance set at 0.05 returns a power of 0.80 with $n=3$.

We have used 6 mice/group in the adoptive transfer experiment. Also in this case no preliminary data were available. If we assume a μ_1 of 2 for Gpnmb⁺ and a μ_2 of 5 for Gpnmb⁻, as suggested by our experiment, with a sigma of 1.7 and a statistical significance at the threshold of 0.05, the power calculation for a statistical significance set at 0.05 returns a power of 0.80 if $n=6$ as in our experiment.

For all experiments a two-sided test was considered. All data were tested for normal distribution and equal variance before performing any statistical analysis using Prism v5 or v6. Power calculation has been made using the free online tool available at <http://www.stat.ubc.ca>

Results

IL6 treatment rescues the phenotype of macrophages deficient for phagocytic cargo digestion *in vitro* and *in vivo*

To investigate pathways regulating the late stages of efferocytosis we tested bone marrow derived macrophages (BMDMs) from Gpnmb⁺ and Gpnmb⁻ mice, that are deficient for the last step of phagocytosis. We analyzed the RNA from Gpnmb⁺ and Gpnmb⁻ BMDMs fed with apoptotic thymocytes (apoT) on a low-density qPCR array for inflammatory cytokines

and chemokines. One of the most downregulated genes in Gpnmb- BMDMs is Il6 (Fig.1A-B and Fig.S1A). Gpnmb- BMDMs are able to initiate efferocytosis but internalization of apoT rapidly tails off (Fig.1C and gating strategy Fig.S1B). Il6 mRNA is increased at 30 min and 60 min after addition of apoT to WT BMDMs (Fig.1D) and IL6 protein levels are constant for up to 120 min (Fig.1E and Fig.S1C). This suggests that IL6 is secreted in a cargo digestion-dependent manner. We hypothesized a role for IL6 in sustaining efficient efferocytosis in macrophages. To this end, we tested, by flow cytometry, the ability of Gpnmb⁺ and Gpnmb- BMDMs to phagocytose apoT in the presence or in the absence of increasing concentrations of recombinant murine (rm)IL6. rmIL6 increases the percentage of Gpnmb- phagocytic BMDMs at any concentration tested (2h duration of efferocytosis, Fig. 1F). To confirm the role of IL6 *in vivo* we induced acute liver damage by single injection of CCl₄ in Gpnmb⁺ and Gpnmb- mice and we treated them with rmIL6 or vehicle 6h prior to culling (Fig.1G). We digested the livers and analyzed the percentage of infiltrating macrophages by flow cytometry (CD45⁺Lin⁻CD11b^{high}F4/80^{low}). In this gate, we analyzed the percentage of phagocytic (PKH26PCL⁺) inflammatory (Ly6C^{high}), or restorative (Ly6C^{low}) macrophages. While rmIL6 treatment did not affect the overall percentage of infiltrating macrophages (Fig.1H and gating strategy in Fig.S1E), it normalized to a wild type pattern the phenotype of Gpnmb- infiltrating inflammatory macrophages *in vivo*. It had less effect on restorative macrophages (Ly6C^{low}) which infiltrate as inflammatory macrophages before undergoing a phenotypic switch (14)(Fig.1I and representative plots Fig.1J).

phosphoSTAT3 (pSTAT3) is an upstream mediator of IL6 in efferocytosis

We then analyzed possible pathways upstream of IL6. Using two inhibitors of NF-κB, we demonstrated that there is no role for this transcription factor in IL6 regulation of efferocytosis (Fig.S1D). IL6 is one of the cytokine pathways known to induce hepatocyte proliferation in models of hepatocellular carcinoma via activation of pSTAT3. In these studies, IL6 is identified as a single factor co-regulating key aspects of macrophage mediated tissue remodelling and phenotype with epithelial regeneration (30, 31). We tested the hypothesis that this pathway sustains macrophage efferocytosis in a cell-autonomous manner in BMDMs. pSTAT3 blockade resulted in impaired phagocytosis in BMDMs 2h after the start of phagocytosis (Fig.2A-B) as assessed by flow cytometry. We confirmed the results using live imaging: Blocking p-STAT3 impairs phagocytosis of zymosan-A coated, pH sensitive (pHrodo) beads in WT BMDMs and in human monocyte derived macrophages alike (Fig.2C-D), showing the conserved nature of this pathway. In WT BMDMs the impairment is evident as soon as 30-35 minutes after the start of phagocytosis, and is more complete than blockade of the acidification of the phagosome with bafilomycin (Fig.2C and movie S1, S2). The pHrodo beads emit fluorescence when in acidic compartments and empty vacuoles are observed in the cytoplasm of BMDMs in the presence of the pSTAT3 blocking peptide (movie S3). A similar but less dramatic effect is observed if pSTAT3 is blocked in BMDMs prior to the induction of phagocytosis, indicating that the pro-phagocytic activity relies mainly on *de novo* phosphorylated STAT3 (Fig.S2A). The blockade of pSTAT3 decreases phagocytosis of infiltrating inflammatory macrophages (Ly6C^{high}) *in vivo* in a model of acute liver damage induced by CCl₄ injection in which a phagocytic cell tracker is injected (PKH26PCL, for Phagocytic Cell Labeling) (Fig.2E). The

percentage of infiltrating inflammatory macrophages positive for PKH26PCL (i.e. performing phagocytosis) is lower in livers from Gpnmb⁺ mice treated with the pSTAT3 blocking peptide as compared to Gpnmb⁺ mice treated with an irrelevant peptide, and similar to livers from Gpnmb⁻ mice (Fig.2F and representative plot in Fig.2G). A similar trend is observed in infiltrating restorative macrophages (Fig.2F). No effect of pSTAT3 inhibition is observed on the overall percentage of resident macrophages (CD45⁺Ly6G⁻CD11b^{low}F4/80^{high}), and infiltrating macrophages in Gpnmb⁻ mice (Fig.2H and Fig.S2B, respectively, and gating strategy in Fig.S1E).

STAT3 phosphorylation is required for the maintenance of efficient efferocytosis

To confirm that IL6 is downstream of pSTAT3, we treated BMDMs with the blocking peptide for pSTAT3, and rescued their phagocytosis using rmIL6. Live imaging results show that rmIL6 is able to rescue BMDMs phenotype at 2h of phagocytosis (Fig.3A-B). The result is confirmed by flow cytometry (Fig.3C). Two hours after the start of efferocytosis *Il6* mRNA is reduced in the presence of the pSTAT3 blocking peptide (Fig.3D); Consistently, IL6 protein shows a reduced trend in the supernatant of phagocytosing BMDMs when pSTAT3 is blocked (Fig.3E). Therefore, pSTAT3 and IL6 appear to be in the same pathway, effective in sustaining phagocytosis, with IL6 controlled at transcriptional level by pSTAT3 when macrophages are exposed to apoptotic cells for more than 60 min. We then explored the dynamics of pSTAT3 activation upon phagocytosis using flow cytometry, in order to interrogate the phagocytic fraction of BMDMs (CD11b⁺CMTMR⁺). pSTAT3 is activated as early as 7 min after the start of phagocytosis and its activation is sustained at any time point analyzed (Fig.3F and Fig.S2D). Gpnmb⁻ BMDMs can internalize the cargo but they cannot digest it. Consistent with a role for the pSTAT3-IL6 axis in sustaining efferocytosis at the cargo digestion stage, pSTAT3 can be activated in Gpnmb⁻ BMDMs but its activation fails to be sustained at later time points (Fig.3G and Fig.S2E) and pSTAT3 intracellular localization is disrupted in Gpnmb⁻ BMDMs (Fig.S2G). However, analysis of pSTAT3 activation during phagocytosis following rmIL6 treatment showed no significant difference (data not shown).

pSTAT3 drives IL10 secretion early after the start of phagocytosis

Later time points of phagocytosis are associated with a cargo digestion-dependent activation of pSTAT3. However, the consequence of the early phosphorylation of STAT3 is thus far unexplored. A target molecule of pSTAT3 is IL10, and links between IL10 and phagocytosis have been previously reported (32, 33). We hypothesized that IL10 could be downstream of pSTAT3 early activation and have a pro-phagocytic role. To this end, we treated Gpnmb⁺ and Gpnmb⁻ BMDMs with rmIL10 and measured their phagocytic ability. Gpnmb⁺ BMDMs showed increased phagocytosis at 15 min and Gpnmb⁻ BMDMs demonstrated a similar trend (Fig.4A-B and Fig.S2F). IL10 protein increased at 15 min in the supernatants of fed BMDMs (Fig.4C), pointing to a possible early non-transcriptional regulation of IL10. To confirm a role for pSTAT3 in the regulation of IL10 secretion early after the start of efferocytosis, we blocked pSTAT3 and measured IL10 protein in the supernatants of fed WT BMDMs. IL10 protein dramatically decreases at 15 min and 30 min (Fig.4D). *Il10* transcription is not induced in BMDMs performing efferocytosis until 30-60 min (Fig.4E), thereby suggesting a non-transcriptional control of IL10 secretion by pSTAT3. Consistently, the levels of *Il10* mRNA are substantially stable when we treat BMDMs with the blocking

peptide for pSTAT3 during efferocytosis (Fig.4F). Thus, pSTAT3 appears to regulate IL10 release at early stages of efferocytosis in a transcriptionally independent manner. Consistent with a role of IL10 at early stages of phagocytosis we did not observe any difference in IL10 levels in the supernatants of Gpnmb⁺ and Gpnmb⁻ macrophages after overnight efferocytosis (Fig.S2H). SOCS3 may play a role in macrophage phagocytosis and polarization. Moreover, SOCS3 is known to support IL6 transcription (34) (35). Consistent with a role of Socs3 in IL6 production and signalling, we found that Socs3 transcription is controlled by pSTAT3 activation and cargo digestion at 1h after the start of phagocytosis in BMDMs (Fig. 4G-H). To show that the role of pSTAT3 is conserved in tissue repair and remodelling across species we used the tail fin injury model in *D. rerio* (zebrafish) embryos. *D. rerio* embryos were harvested 48h post-fertilization and we performed the tail injury at the level of the notochord. First, we replicated recent data suggesting that blocking the acidification of intracellular compartments delays tail fin regeneration, a defect similar to that of Gpnmb⁻ mice (18, 36). To this end, embryos were left to recover from tail fin injury in the presence or in the absence of bafilomycin to block the acidification of intracellular compartments; length and area of the regenerating fin were recorded at 24h, 48h and 72h post damage. Treated embryos recapitulated the phenotype of the Gpnmb⁻ mice with a lower regeneration of the tail fin at 72h post-injury (data not shown). To test the hypothesis that tail fin remodelling and regeneration is pSTAT3-dependent we repeated the same experiment monitoring the re-growth of the tail fin in the presence or absence of a small molecule inhibiting p-STAT3, 5'-15' DPP (Fig.S4F). Reduced tail fin remodelling and regeneration was evident at 24h after the injury induction. By 48h and 72h after injury the tail fin remodelling and regeneration was dramatically impaired in terms of both length and area (Fig.S4G-H). Whilst we cannot prove that the blockade to tail fin regeneration is macrophage-mediated, our data provide a link between pSTAT3 and tissue repair in both zebrafish and mice.

Digestion of the phagocytic cargo limits tissue damage, regulates tissue proliferation and controls macrophage phenotype in a model of chronic sterile liver injury

Sterile liver injury has proven a useful model to define the interplay of parenchymal cells and inflammation in the mammalian wound healing response. Therefore, we proceeded to investigate the potential role of cargo digestion during efferocytosis as a regulator of tissue damage and macrophage phenotype in a model of chronic sterile liver injury. We treated Gpnmb⁺ and Gpnmb⁻ mice with CCl₄ for 6 weeks to induce iterative liver parenchymal sterile necrosis and inflammation with resulting liver fibrosis (Fig.5A). At the suspension of CCl₄ administration we performed a time course analysis of circulating plasma alanine transaminase (pALT), aspartate transaminase (pAST) as markers of liver damage, together with plasma alkaline phosphatase (pALP), albumin, urea and creatinine (Fig.5B-C). The liver/body weight ratio was lower in Gpnmb⁻ mice at 72h, while the two groups of mice show a comparable weight increase during the 6 weeks of treatment (Fig.S3A-B). pALT and pAST are specifically increased in Gpnmb⁻ mice at 48h after the suspension of the CCl₄ and the trend is maintained at 72h. At one week, the levels of ALT and AST are comparable in the two groups of mice. pALP, albumin, urea and creatinine are unchanged in either genotype at any time points analyzed (Fig.5B-C). We then analyzed the infiltrating macrophages in the damaged liver and the circulating monocytes by flow cytometry (gating

strategy in Fig.S3C). Failure of phagocytic cargo processing does not affect the general percentage of infiltrating macrophages (live CD45⁺Ly6G⁻CD11b^{high}F4/80^{low}) at basal level and at any time point studied (Fig.5D and Fig.5H). We then analyzed the percentage of restorative macrophages (which we and others have shown can be identify as Ly6C^{low} (14)). In Gpnmb⁻ livers, the percentage of Ly6C^{low} infiltrating macrophages was reduced relative to Gpnmb⁺ controls at any time points analyzed (Fig.5E-F), suggesting that phagocytosis is a driver of the switch to a Ly6C^{low} restorative macrophage phenotype. Analysis of the expression of liver cytokines and chemokines in the whole liver showed unchanged levels of *Mcp1/Ccl2*, *Cxcl12* and *Hmgbl* (Fig.S3D). Consistent with the lack of difference in the overall infiltration of macrophages, T lymphocyte infiltrate was comparable in the two groups of mice at all time points analyzed and at basal level (Fig.5G). Circulating granulocytes, monocytes and, particularly, classic monocytes were similar in the two groups of mice (Fig. 5I). Overall, the impairment in phagocytic cargo processing caused an increased liver damage and prevented macrophage phenotype switching during regeneration. If IL6 has a pro-phagocytic role relevant for the clearance of damaged cells, then we would expect an increase in damage in the livers of IL6KO mice with iterative sterile injury (Fig. 5J). Supporting this model and directly reproducing the phenotype observe in Gpnmb⁻ mice, IL6KO mice showed higher levels of circulating ALT and AST at 48h after CCl₄ withdrawal, without showing any difference in the level of pALP (Fig.5K) or in their liver:body weight ratio (Fig.S4A).

We then verified the impact of impaired phagocytic cargo processing on liver pathology. At one week of recovery, there is no difference in fibrosis between the two groups of mice (Fig. 6A-B). Gpnmb⁻ BMDMs showed similar level of Tgf- β when compared to Gpnmb⁺ BMDMs after overnight of phagocytosis (Fig. 6C). Further, the lack of difference in liver fibrosis between the two groups of mice was confirmed by collagen 1, Tgf- β , Mmp-2, and Mmp-7 mRNA analysis (Fig. 6D-F, I and L). Col3a1 mRNA was increased at 48h (Fig.6G), but no differences between the two groups of mice was observed in the immunohistochemical analysis (Fig.6H). However, Gpnmb⁻ livers show increased α -SMA levels at 48h of recovery (Fig. 6J-K). The similar levels of liver fibrosis in Gpnmb⁻ mice may result in part from the increased proliferation of parenchymal cells at 48h of recovery (Fig.6M-N).

Digestion of the phagocytic cargo limits tissue damage in a model of acute liver injury by acetaminophen overdose, and IL6 treatment improves phagocytosis of infiltrating macrophages

We collected further evidence for a correlation between phagocytosis, IL6 and damage resolution in a clinically relevant model of sterile acute liver damage by acetaminophen (APAP) intoxication (Fig.7A), that is characterized by extensive parenchymal cell death and inflammation (37, 38). Gpnmb⁻ mice have higher level of plasma (p)ALT at 8h post-injury (Fig.7B); consistently, they showed a more extended necrotic area than their Gpnmb⁺ counterparts as assessed by image analysis (Fig.7C-D and Fig. S3E). We then dosed C57Bl/6 WT mice with APAP and treated them with rmIL6 at the same time as, or two hours after, the APAP injection.. To track phagocytic cells we injected PKH26PCL in some of the mice; mice which were culled at 8h from APAP dosing to quantify the effect of rmIL6

treatment on the phagocytic ability of infiltrating macrophages (Fig.7E). We did not observe a significant effect of IL6 treatment on the necrotic area, the pALT or the liver:body weight ratio (Fig.7F-G and Fig.S4B). Importantly, the number of phagocytic macrophages/g liver was increased in rmIL6 injected mice (Fig.7H-I). The treatment was efficacious in increasing macrophage phagocytosis without affecting the percentage of infiltrating macrophages and granulocytes in the damaged liver (Fig.7J-K). The local effect of the systemic treatment with rmIL6 is confirmed by a lack of effect on the phagocytic ability of circulating monocytes (Fig.7L).

Phagocytosis drives the conversion of inflammatory into restorative macrophages

To test the hypothesis that pro-phagocytic positive feedback loop triggered by the STAT3-IL6 axis drives the conversion of macrophage phenotype, and thereby provides a link between tissue damage and tissue repair, we administered rmIL6 to Gpnmb⁺ and Gpnmb⁻ mice after induction of chronic liver injury by CCl₄. Mice were culled at 48h and 72h after CCl₄ withdrawal (Fig.8A). rmIL6 restored the ability of Gpnmb⁻ mice to convert the inflammatory Ly6C^{high} macrophages into restorative Ly6C^{low} macrophages within 72h of administration (Fig.8B-C). Consistent with a reprogramming action of phagocytosis, WT BMDMs fed with CMTMR-labelled apoptotic thymocytes showed a sharp decrease of the percentage of Ly6C^{high} events; i.e. *bona fide* inflammatory BMDMs were reduced just as the percentage of phagocytic BMDMs increased in the population (Fig.8D-E). Independent evidence was sought by treating APAP dosed mice with rmIL6 at distinct time points (Fig. 7E). The number of Ly6C^{high} infiltrating macrophages (i.e. infiltrating inflammatory macrophages) was lower in mice treated with rmIL6 (Fig.8F), which also showed a higher number of phagocytic macrophages infiltrating the liver (Fig.7H). To demonstrate that phagocytosis triggers a macrophage phenotypic switch we induced acute damage by a single injection of CCl₄ in C57Bl/6 mice. Three hours later, we adoptively transferred CFSE⁺Gpnmb⁺ and CMTMR⁺Gpnmb⁻ BMDMs separately, or mixed in 1:1 ratio. Five hours later mice are culled, the liver digested and cells analyzed (Fig.9A, gating strategy in Fig.S4C). Macrophage conversion to a restorative phenotype was monitored using the level of Ly6C expression. The percentage of Ly6C^{high} adoptively transferred macrophages was calculated in Gpnmb⁺ and Gpnmb⁻ BMDMs before and after transplant. Both genotypes showed a lower post-transfer percentage of Ly6C^{high} transferred macrophages as compared with pre-transfer (Fig.9B). However adoptively transferred Gpnmb⁻ macrophages showed a consistently higher percentage of Ly6C^{high} cells vs. their Gpnmb⁺ counterparts co-transferred in the same mouse and therefore in an identical microenvironment (Fig.9B-D). The higher percentage of Ly6C^{high} cells in Gpnmb⁻ macrophages was also confirmed when the data from the mice receiving only Gpnmb⁺ or Gpnmb⁻ BMDMs were included in the analysis (Fig.9E-F). The transfer of Gpnmb⁻ BMDMs reduced the hepatic recruitment of the endogenous macrophages: host livers showing a lower number of both Ly6C^{high} and Ly6C^{low} infiltrating macrophages and a contraction of the Ly6C^{low} population (Fig.9H). The difference observed was not due to a difference in the viability of extracted cells (Fig.9G and Fig.S4D-E).

Discussion

Our data suggest that the processing of apoptotic cells activates a STAT3-IL10-IL6 autocrine-paracrine loop, enabling macrophages to maintain their scavenging ability. Furthermore, a failure of phagocytic cargo processing results in the reduced uptake of debris (predominantly in pro-inflammatory macrophages), prolonged liver damage, enhanced hepatocyte proliferation and a reduced expression of IL10. These data suggest that macrophage ingestion of apoptotic cell and IL6 production are important for debris clearance and the phenotypic switch to a restorative phenotype. Apoptotic cells are necessary to trigger an IL4/IL13-dependent tissue repair response in models of helminth infection (39). Here, for the first time we provide data linking an immediate event in pro-inflammatory macrophages to the programming of tissue remodelling and regeneration in the context of sterile injury. Phagocytosis of apoptotic bodies (efferocytosis) may require the presence of autophagy-related molecules for the assembly of the phagosome, a process named LC3-associated phagocytosis (LAP) (40). During LAP some of the autophagy machinery, including LC3, is recruited to pathogen, apoptotic and necrotic cell-containing phagosomes; as a result optimal degradation of the phagocytosed cargo is achieved (41, 42). Gpnmb is an interactor of LC3 (18), making Gpnmb- mice a useful model to study the effect of an incomplete phagocytic process of apoptotic cells on sterile injury and repair models.

In our model, STAT3, IL10 and IL6 are in the same pathway and provide mechanistic insight on previous observations that correlated IL10 and phagocytosis in *in vitro* models (32, 33, 43, 44). Our work provides a mechanistic explanation to recent data regarding the restorative role of Gpnmb⁺ macrophages in acute liver (45) and kidney injuries (18), and in DSS-induced colitis (46); and the pro-resolution role of Gpnmb⁺ macrophages in a model of peritonitis (47). Growing evidence correlates pSTAT3 activation with tumor cell proliferation, genomic instability, and migration (48–51) (52, 53). In addition, recent studies establish a link between pSTAT3 activation and immunomodulation in tumors, via infiltrating monocytes switching phenotype to tumor-associated macrophages (54–57). Moreover, the IL10-IL6-pSTAT3 pathway has been reported having a role in the inflammatory process in inflamed adipose tissue from obese patients (58). Our data suggests pSTAT3 acts as a driver of inflammatory macrophage phagocytosis and phenotypic conversion. Whilst the switch to an anti-inflammatory, pro-remodeling phenotype is detrimental in tumor models (11, 59), it is required in physiological tissue repair (1, 9). The completion of a correct phagocytic process leads macrophages to switch their inflammatory phenotype into a restorative one, evidencing that phagocytosis is a major process through which the initial damage phase initiates the repair of the tissue. Our *in vitro* data and *in vivo* adoptive transfer show this process happens early after the induction of damage. The identification of phagocytosis as a key element mediating macrophage phenotype conversion is of therapeutic relevance for a number of diseases. Means to increase restorative-like macrophage phenotype have proved to be beneficial to block central nervous system inflammation (60), systemic lupus erythematosus (61) and to contribute to hepatic progenitor cell specification in models of liver disease (16, 62). STAT3-IL6 is a pro-proliferative signal in hepatocellular carcinoma (HCC) development. Tumor-associated macrophages in HCC have a phenotype similar to restorative macrophages (63).

Macrophages forced to stay in an inflammatory phenotype downregulate Stat3 and have a higher anti-HCC activity (64). Our data linking a STAT3-IL10-IL6 positive feedback loop with phagocytosis enhancement and a macrophage phenotypic switch may also explain recent data showing that IL6 producing macrophages, polarised with rIL4, block neuro-inflammation *in vivo* (65). The possible link between STAT3-IL10-IL6 and the control of hepatocyte proliferation is worthy further investigation. Macrophages link tissue necrosis and repair in many diseases; explaining how to program their regenerative response via the control of their ability to scavenge dead cells will be important for future therapeutic targeting in multiple clinical settings, which may include acute liver injury and chronic liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

SLE	Systemic Lupus Erythematosus
CCl₄	Carbon Tetrachloride
hMDMs	Human monocyte derived macrophages
Gpnmb	Glycoprotein Non-Metastatic Melanoma B
APAP	Acetaminophen
SD	Standard Deviation
LC3	Light Chain 3
NPC	Non-Parenchymal Cell
apoT	Apoptotic thymocytes

BMDMs	Bone marrow-derived macrophages
IF	Immunofluorescence
pALT	plasma ALT
pAST	plasma AST
pALP	plasma ALP
pSTAT3	phosphoSTAT3
H&E	Hematoxylin and eosin
LAP	LC3-associated phagocytosis

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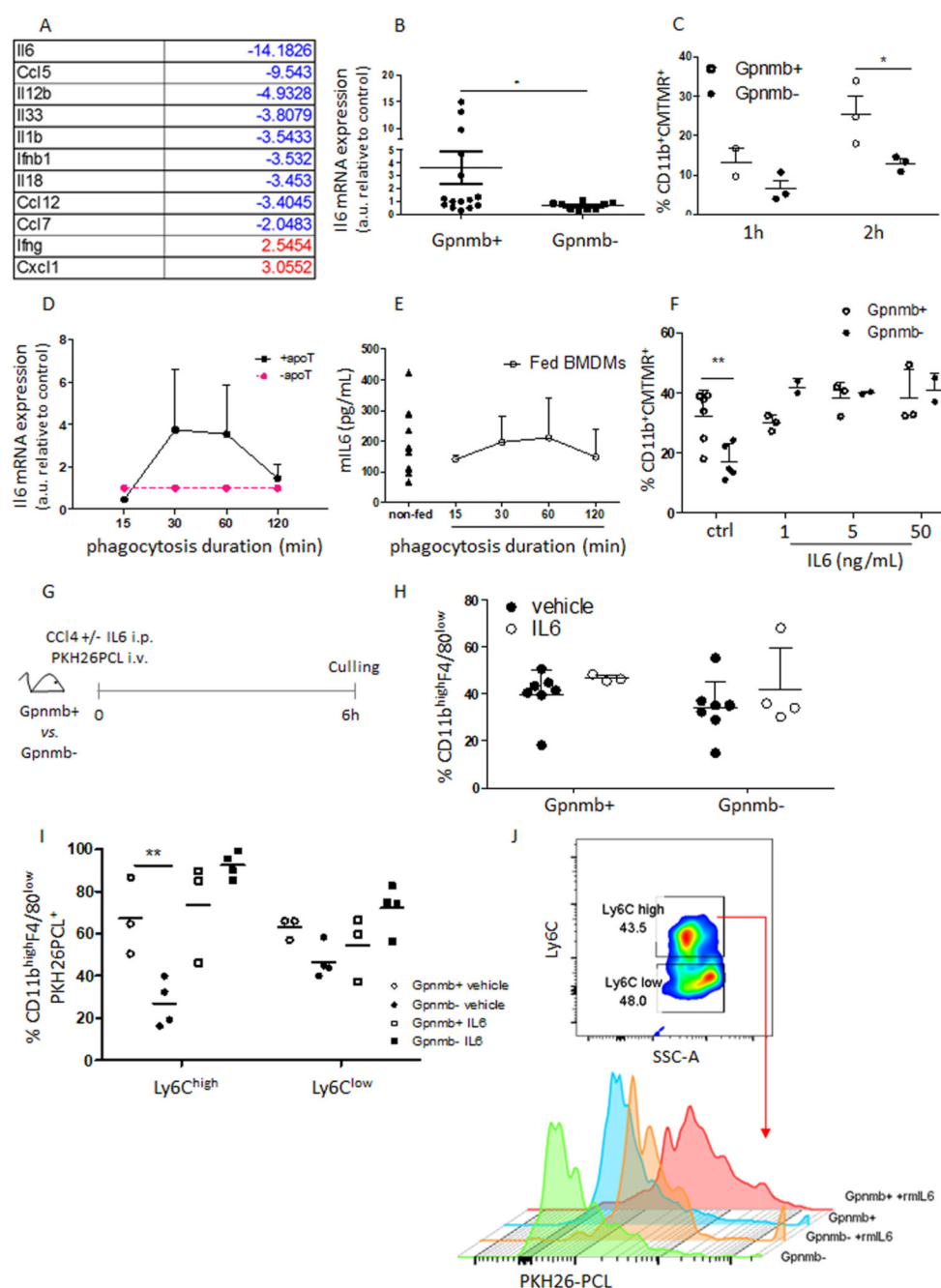


Figure 1. IL6 treatment rescues the phenotype of macrophages deficient for phagocytic cargo digestion *in vitro* and *in vivo*

A. Low-density qPCR array analysis has been performed on mRNA extracted from Gpnmb⁺ and Gpnmb⁻ BMDMs fed with apoptotic thymocytes for an overnight (o.n., n=3). Data are expressed as relative expression of the average of the expression of each gene in the sample group (Gpnmb⁻) vs. control group (Gpnmb⁺). A representative list of the most regulated genes is reported. In blue are the most downregulated genes, in red the genes upregulated of >2 folds in the sample vs. control group. The full list of genes analyzed is provided in SI.

B. Il6 mRNA analysis on RNA from BMDMs fed with apoptotic thymocytes at a phagocyte:target ratio of 1:5 for an o.n. Gene expression has been calculated using the $\Delta\Delta CT$ method relative to housekeeping gene β -actin. The graph reports the ratio between the $\Delta\Delta CT$ of the fed cells with the $\Delta\Delta CT$ of one of the control, untreated Gpnmb+ BMDMs. Data have been analyzed with Student's T-test for unpaired data. * $p < 0.05$

C. Percentage of phagocytic Gpnmb+ (white dots) and Gpnmb- (black dots) BMDMs fed with CMTMR labelled apoptotic thymocytes. Each dot represents a biological replicate.

D. Il6 mRNA dosage on RNA from BMDMs fed with apoptotic thymocytes (apoT) at a phagocyte:target ratio of 1:5 for the indicated time point (black solid line) or not fed with apoT (pink dotted line). Gene expression has been calculated using the $\Delta\Delta CT$ method relative to housekeeping gene β -actin. The graph reports the ratio between the $\Delta\Delta CT$ of each time point with the $\Delta\Delta CT$ of one of the control, untreated BMDMs at the same time point.

E. IL6 protein has been analyzed in the supernatant of the same BMDMs at the indicated time points. Results are expressed as pg/mL. Average $\pm$ SD of three independent experiments is reported. Unfed BMDMs: each triangle equals a supernatant from one well of unfed macrophages. Results of three independent experiments are pulled.

F. Percentage of phagocytic Gpnmb+ (white dots) and Gpnmb- (black dots) BMDMs fed with CMTMR-labelled apoptotic thymocytes in the presence or in the absence of rmIL6 at the indicated concentration for 120 minutes. Each dot represents a biological replicate.

G. Experimental design: Gpnmb+ and Gpnmb- mice have been injected i.p. with a single CCl_4 injection and with rmIL6 or saline. PKH26PCL has been injected at the time of damage via tail vein to track phagocytic cell *in vivo*. Mice are culled after 6h and liver is harvested, digested and labelled for flow cytometry analysis.

H. Flow cytometry analysis of liver digest. Infiltrating macrophages have been identified as described in Fig.S1. Each dot represents the analysis of one liver digest. Black dots = vehicle (PBS) treated mice. Empty dots = rmIL6 treated mice

I-J. Flow cytometry analysis of liver digest. The percentage of phagocytic macrophages is calculated as percentage of PKH26PCL⁺ events in the gate of Ly6C^{high} and Ly6C^{low} events (representative density plot and histograms in **L**).

I. White dots: Gpnmb+ mice treated with CCl_4 and vehicle (PBS). Black dots: Gpnmb- mice treated with CCl_4 and vehicle. White square; Gpnmb+ mice treated with CCl_4 and rmIL6. Black square: Gpnmb- mice treated with CCl_4 and rIL6. Each dot is a distinct mouse. Mean is reported.

D-F, H, I. Data have been analyzed with one-way or two-way ANOVA for unpaired data when appropriate followed by a Bonferroni post-hoc test to compare all pairs of columns. Data have been checked for equal variance before further analysis. * $p < 0.05$ ** $p < 0.01$

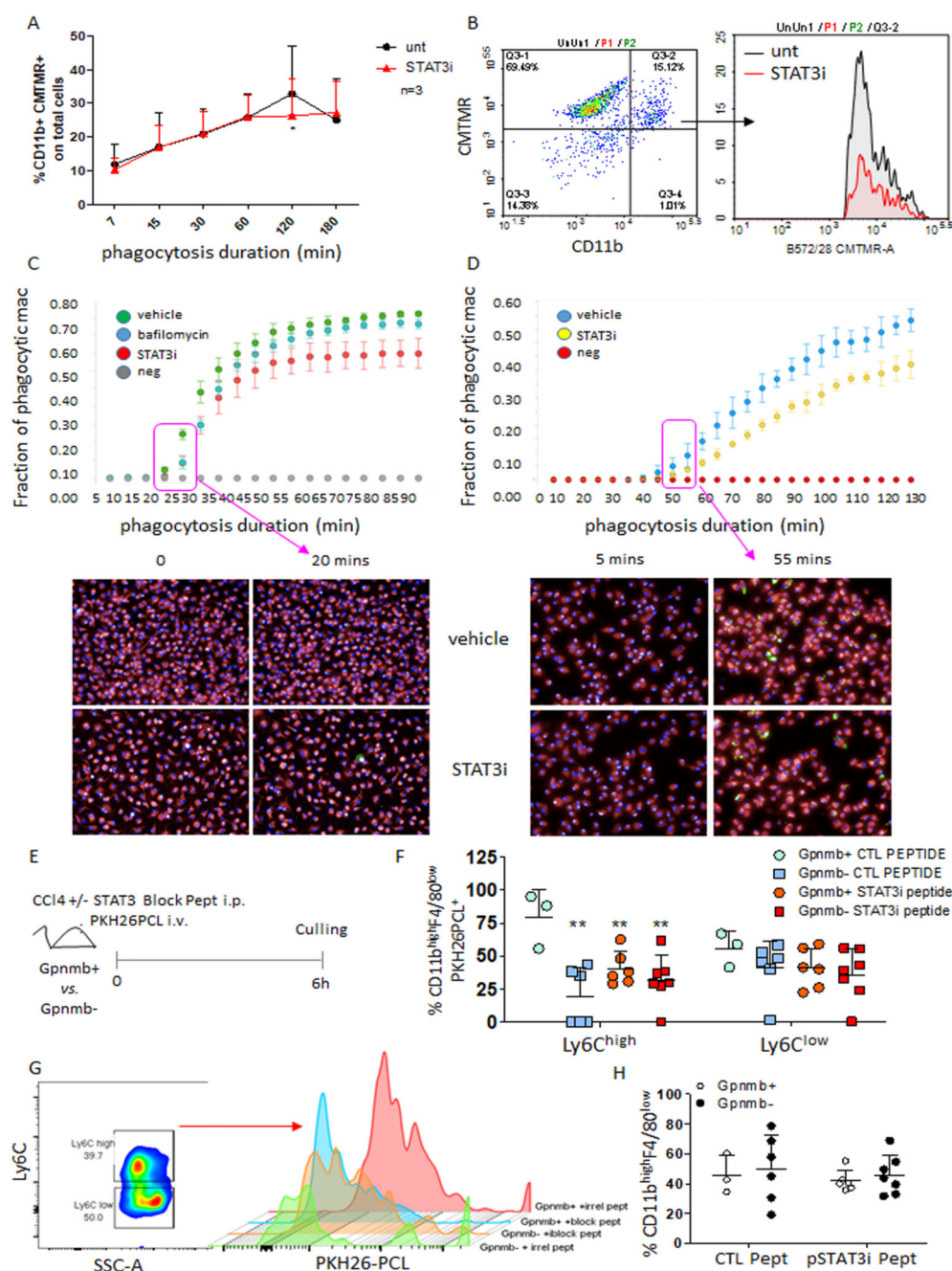


Figure 2. pSTAT3 is upstream IL6 effect in the control efferocytosis

A. BMDMs fed with CMTMR-labelled apoT in the presence of vehicle (DMSO) or pSTAT3 (Tyr506) inhibitory peptide at various time points after adding apoT. n=3, average $\pm$ SD is reported. Data have been analyzed with two-way ANOVA for unpaired data followed by Bonferroni post-hoc test to compare all pair of columns. Data have been checked for equal variance before further analysis. *p<0.05; **p<0.01.

B. Representative dot plot and histogram plot for the analysis carried out in A.

C. Live imaging of BMDMs phagocytosing pH-sensitive (pHrodo) beads. Results are expressed as proportion of phagocytic cells on total number of cells. Mean and SD at the indicated time points are reported for the distinct treatment groups. Green dots: BMDMs; Blue dots: BMDMs+bafilomycin; Red dots: BMDMs+p-STAT3 inhibitory peptide. In the bottom panel representative pictures from the live imaging are reported.

D. Live imaging of phagocytic hMDMs: results are expressed as proportion of phagocytic cells on total number of cells. Mean and SD are reported. Red dots: negative control, unfed cells; Blue dots: hMDMs treated with vehicle; Yellow dots: hMDMs treated with p-STAT3 inhibitor. In the bottom panel representative pictures from the live imaging are reported.

E. Experimental design: Gpnmb⁺ and Gpnmb⁻ mice have been injected i.p. with CCl₄ once together with a pSTAT3 (Tyr506) blocking peptide or an irrelevant peptide. PKH26PCL has been injected at the time of damage via tail vein to track phagocytic cell. Mice are culled after 6h and liver is harvested, digested and labelled for flow cytometry analysis.

F-G. Flow cytometry analysis of liver digest. Infiltrating macrophages have been identified as described in Fig.S1 and the % of phagocytic macrophages is calculated as % of PKH26PCL⁺ events in the gate of inflammatory (Ly6C^{high}) and restorative (Ly6C^{low}) macrophages. Light blue dots: Gpnmb⁺ mice treated with CCl₄ and irrelevant peptide. Blue squares: Gpnmb⁻ mice treated with CCl₄ and irrelevant peptide. Orange dots: Gpnmb⁺ mice treated with CCl₄ and pSTAT3 blocking peptide. Red squares: Gpnmb⁻ mice treated with CCl₄ and pSTAT3 blocking peptide. Each dot is a distinct mouse. Mean and SD are reported.

G. Representative histogram plot of PKH26PCL fluorescence in the gate of infiltrating inflammatory macrophages from livers of Gpnmb⁺ (left panel) and Gpnmb⁻ (right panel) mice.

H. Flow cytometry analysis of liver digest. Infiltrating macrophages have been identified as described in Fig. S1. Each dot represents the analysis of one liver digest. White dots = Gpnmb⁺. Black dots = Gpnmb⁻.

F and H. Data have been analyzed with two-way ANOVA for unpaired data followed by a Bonferroni post-hoc test to compare all pair of columns. The distribution of the data has been checked for equal variance before applying any statistical test. *p<0.05; **p<0.01

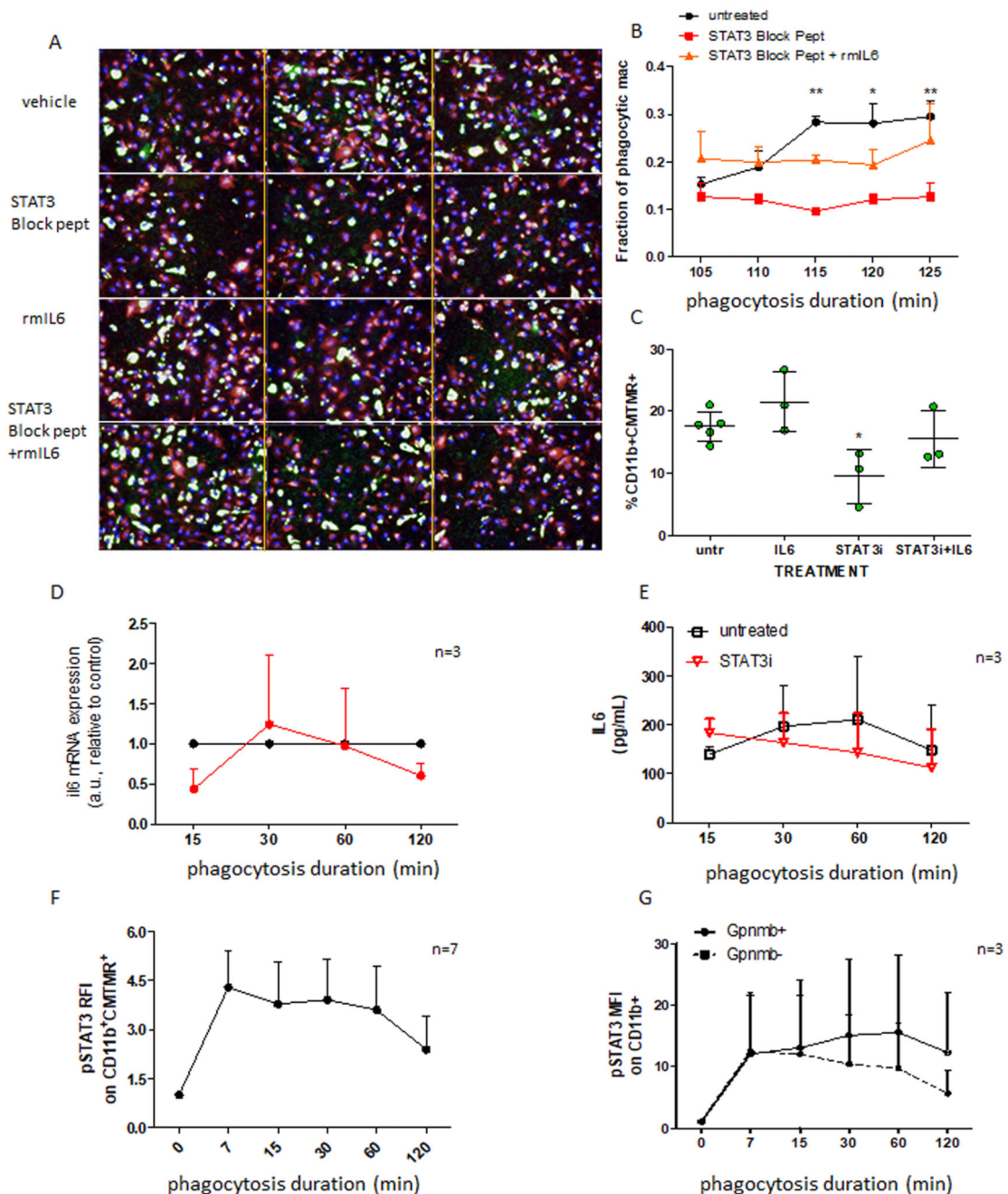


Figure 3. STAT3 phosphorylation is required for the maintenance of an efficient efferocytosis.

A. Representative pictures from the live imaging are reported. Each picture is an independent well. Red = macrophage cytoplasm; Blue = nucleus; Green = phagocytosed zymosan A-coated beads.

B. Live imaging of BMDMs phagocytosing pH-sensitive (pHrodo) beads. Average $\pm$ SD at the distinct time points are reported for the distinct treatment groups. Black dots: untreated BMDMs; Red dots: BMDMs+pSTAT3 inhibitory peptide; Orange dots: BMDMs+pSTAT3 inhibitory peptide+rmIL6.

C. Flow cytometry analysis of phagocytosis in untreated vs. rmIL6 50ng/mL, pSTAT3(Y705) blocking peptide (STAT3i) 30uM or rmIL6 50ng/mL and pSTAT3(Y705) blocking peptide (STAT3i) 30uM treated BMDMs

D. Il6 mRNA analysis on RNA from BMDMs fed with apoT and treated with the pSTAT3 inhibitory peptide at the indicated time points (solid red line) or treated with the vehicle (solid black line). Gene expression has been calculated using the $\Delta\Delta CT$ method relative to housekeeping gene 18s. The graph reports the ratio between the $\Delta\Delta CT$ of each time point with the $\Delta\Delta CT$ of one of the control, untreated BMDMs at the same time point.

E. IL6 protein has been analyzed in the supernatant of the same BMDMs using a multiplex platform at the indicated time points. Results are expressed as pg/mL. Average $\pm$ SD of three independent experiments is reported.

F. pSTAT3 expression after phagocytosis of apoT at distinct time points is analyzed by flow cytometry; the Average $\pm$ SD of the relative fluorescence intensity is plotted. Relative fluorescence intensity is calculated as mean fluorescence intensity (MFI) of the sample divided by the MFI of the control (non-phagocytic naïve BMDMs).

G. pSTAT3 expression after phagocytosis of apoT at distinct time points is analyzed by flow cytometry in Gpnmb+ and Gpnmb- BMDMs; the mean and SD of the relative fluorescence intensity is plotted. Relative fluorescence intensity is calculated as MFI of the sample divided by the MFI of the control (non-phagocytic Gpnmb+ and Gpnmb- BMDMs respectively).

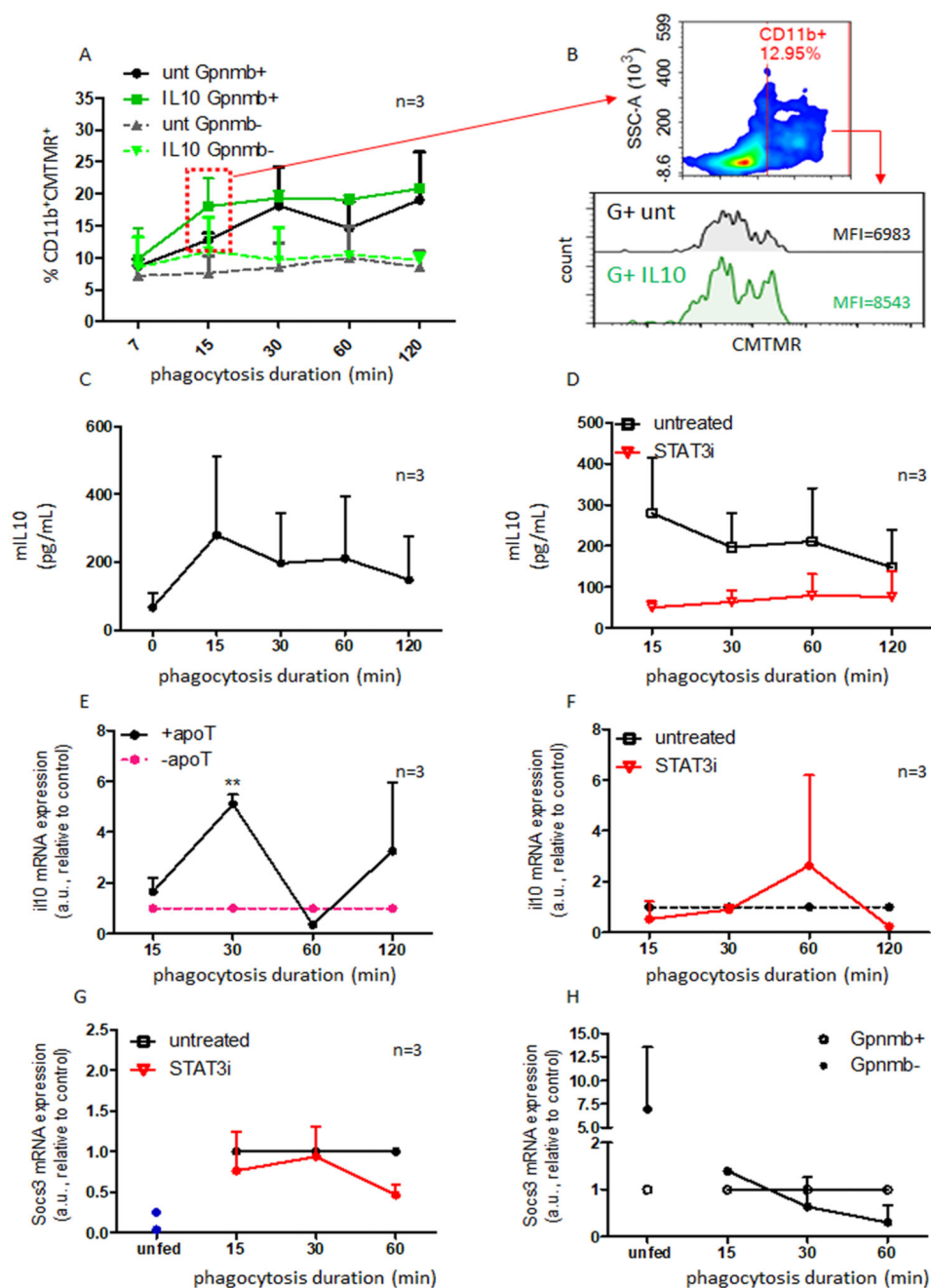


Figure 4. pSTAT3 drives IL10 secretion early after the start of phagocytosis.

A. Gpnmb⁺ (solid lines) and Gpnmb⁻ (dotted lines) BMDMs fed with CMTMR-labelled apoT in the presence of vehicle (DMSO, black and grey lines) or rmIL10 (green lines) at various time points after adding apoT. n=3, average $\pm$ SD is reported.

B. Representative plots of CMTMR MFI analyzed in Gpnmb⁺ BMDMs (CD11b⁺) treated or not with rmIL10 at 15' of efferocytosis (red dotted box in Fig. 4A). Further plots are reported in Fig. S4A.

C. IL10 protein has been analyzed in the supernatants of the same BMDMs at the indicated time points. Results are expressed as pg/mL. Average $\pm$ SD of three independent experiments is reported.

D. IL10 protein has been analyzed in the supernatants of the same BMDMs treated or not with the pSTAT3 blocking peptide (STAT3i) at the indicated time points. Results are expressed as pg/mL. Average $\pm$ SD of three independent experiments is reported.

E. Il10 mRNA dosage on RNA from BMDMs fed with apoptotic thymocytes (apoT) at a phagocyte:target ratio of 1:5 for the indicated time point (black solid line) or not fed with apoT (pink dotted line). Gene expression has been calculated using the $\Delta\Delta$ CT method relative to housekeeping gene β -actin.

F. Il10 mRNA dosage on RNA from BMDMs fed with apoT and treated with the pSTAT3 inhibitory peptide (STAT3i) at the indicated time points (red solid line) or treated with the vehicle (black dotted line). Gene expression has been calculated using the $\Delta\Delta$ CT method relative to housekeeping gene 18s.

G. Socs3 mRNA dosage on RNA from BMDMs fed with apoT and treated with the pSTAT3 inhibitory peptide (STAT3i) at the indicated time points (red line) or treated with the vehicle (black line). Blue dots: non-fed BMDMs. Gene expression has been calculated using the $\Delta\Delta$ CT method relative to housekeeping gene Gapdh.

H. Socs3 mRNA dosage on RNA from Gpnmb⁺ and Gpnmb⁻ BMDMs fed with apoT and treated at the indicated time points. Gene expression has been calculated using the $\Delta\Delta$ CT method relative to housekeeping gene Gapdh.

E-H. The graph reports the ratio between the $\Delta\Delta$ CT of each time point with the $\Delta\Delta$ CT of one of the control (untreated or WT) BMDMs at the same time point.

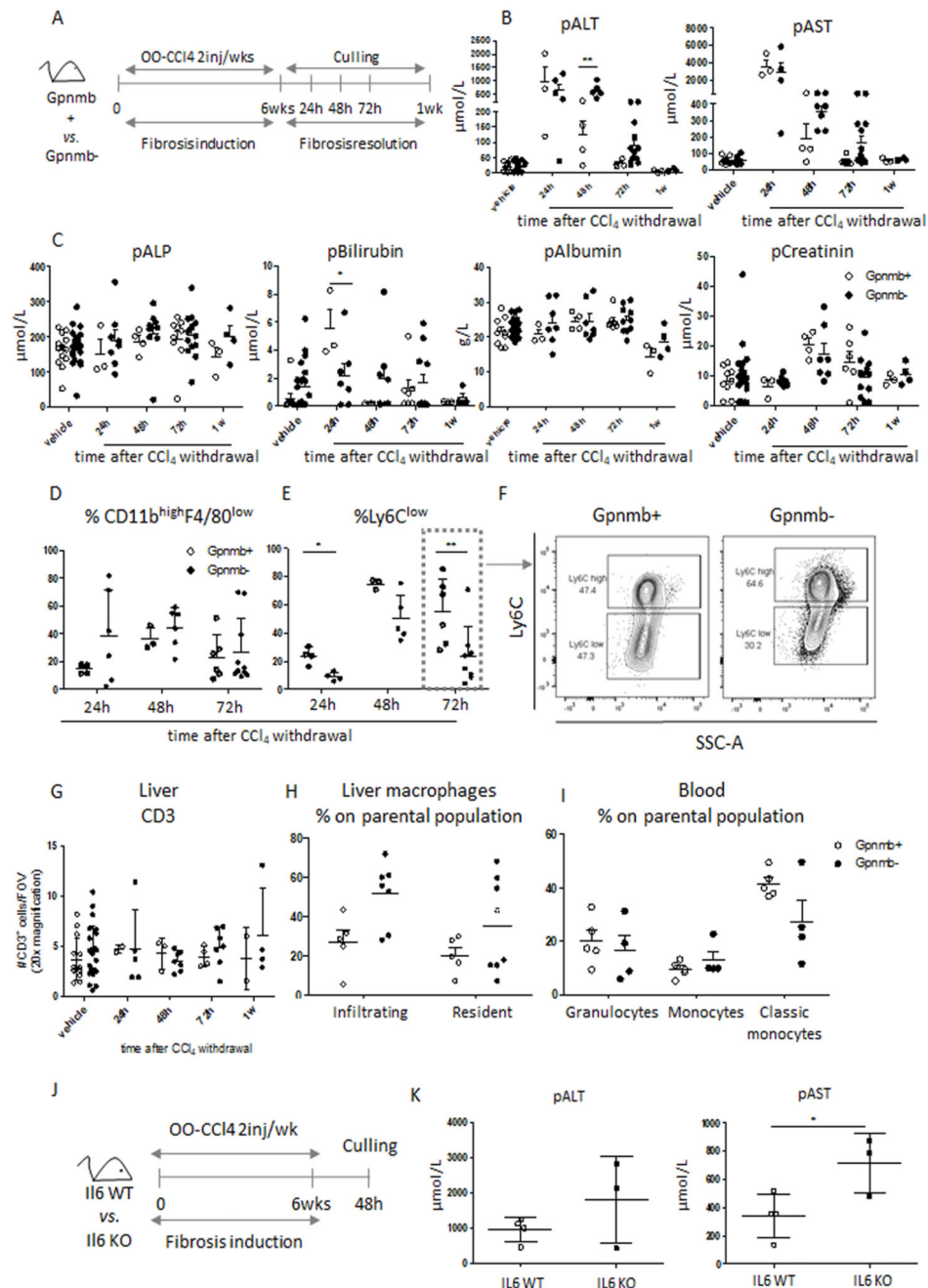


Figure 5. Digestion of the phagocytic cargo limits tissue damage and controls macrophage phenotype in a model of chronic sterile liver injury

A. Experimental design. Gpnmb+ (empty dots) and Gpnmb- (black dots) mice have been treated with CCl₄ twice a week for 6 weeks. CCl₄ administration was then discontinued and mice have been culled at 24h, 48h, 72h and 1 week (1w).

B. plasma (p)ALT, pAST dosages at the indicated culling time point in Gpnmb+ (empty dots) and Gpnmb- (black dots) mice.

C. pALP, pBilirubin, pAlbumin, pCreatinin at the indicated culling time point in Gpnm⁺ (empty dots) and Gpnm⁻ (black dots) mice. Further characterization of the model is reported in Fig.S3 and Fig.6

D. Analysis of the percentage (%) of infiltrating macrophages (CD45+Ly6G-CD11b^{high} F4/80^{low}) in the liver digest of Gpnm⁺ (empty dots) and Gpnm⁻ (black dots) mice at the indicated time points after the discontinuation of the CCl₄ injection.

E. Analysis of the percentage (%) of restorative macrophages (Ly6C^{low}), gated on infiltrating macrophages, in the liver digest of Gpnm⁺ (empty dots) and Gpnm⁻ (black dots) mice at the indicated time points after the discontinuation of the CCl₄ injection.

F. Representative contour plot of the Ly6C expression in infiltrating macrophages in the liver digest of Gpnm⁺ and Gpnm⁻ mice at 72h after the discontinuation of the CCl₄ injection (red dotted box in Fig.4E).

G. CD3 immunostaining on sections of liver of Gpnm⁺ (empty dots) and Gpnm⁻ mice (black dots) treated with CCl₄ for 6 weeks at distinct time point of recovery and of the same mice treated with olive oil only (vehicle). The number (#) of CD3+ T cells is quantified counting CD3+ nuclei/field of view (FOV) using a 20x magnification. 20-30 fields/mouse are counted and averaged. Each dot represents the average of one mouse.

H. Analysis of the percentage (%) of infiltrating (CD45+Ly6G-CD11b^{high} F4/80^{low}) and resident (CD45+Ly6G-CD11b^{low} F4/80^{high}) macrophages in the liver digest of Gpnm⁺ (empty dots) and Gpnm⁻ (black dots) mice treated with olive oil only (basal level).

I. Analysis of the percentage (%) of granulocytes (CD45+Ly6G+CD11b⁺), monocytes (CD45+Ly6G-CD11b+CD115⁺), and classic monocytes (CD45+Ly6G-CD11b+CD115+Ly6C^{high}) in the circulation of Gpnm⁺ (empty dots) and Gpnm⁻ (black dots) mice treated with olive oil (vehicle) only.

L. Experimental design. WT and IL6KO mice have been treated with CCl₄ twice a week for 6 weeks. CCl₄ administration was then discontinued and mice have been culled at 48h.

M. plasma (p)ALT, pAST and pALP dosages at 48h in IL6WT and IL6KO mice.

A-E, G-I and L. Each dot represents a mouse. Average±SD is shown. Data have been analyzed with two-way ANOVA or Student t-test for unpaired data when appropriate. Two-way ANOVA is followed by a Bonferroni post-hoc test to compare all pairs of columns. Data have been checked for equal variance before further analysis. *p<0.05 **p<0.01.

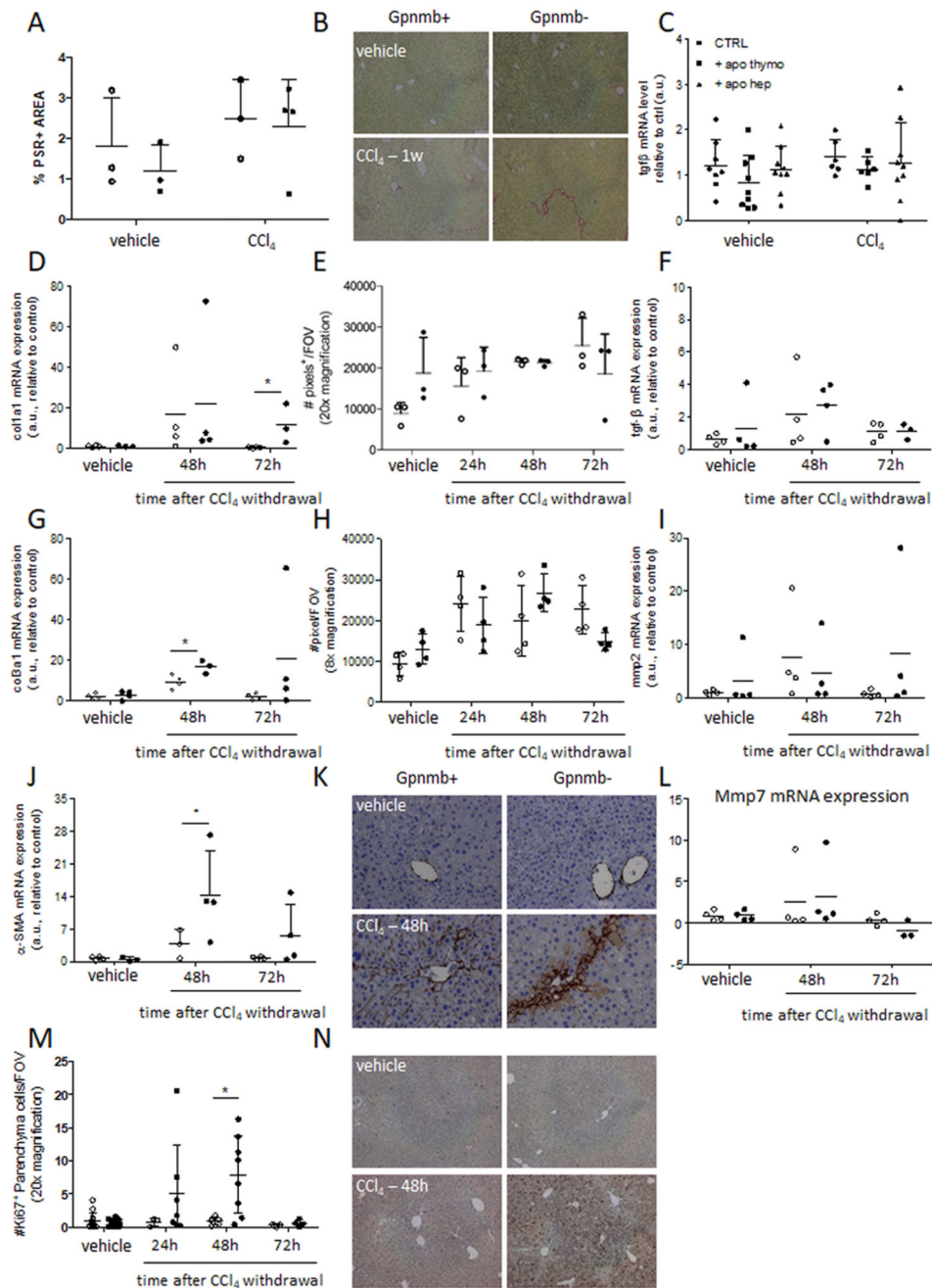


Figure 6. Digestion of the phagocytic cargo increase hepatocyte proliferation and not fibrosis in a model of chronic sterile liver injury.

A. Fibrosis in the regenerating liver is evaluated at 1 week (1w) after the suspension of the CCl₄ using Sirius-Red staining. Fibrotic are distinguished via image analysis and quantified. 20x magnification. Min. 20 field/mouse are quantified and averaged. Each dot represents a mouse.

B. Representative pictures of the liver of Gpnmb+ and Gpnmb- mice left untreated or at 1 week of recovery after CCl₄ discontinuation.

C. Gpnmb⁺ and Gpnmb⁻ BMDMs are fed with apoptotic thymocytes or hepatocytes (1:5 ratio, BMDM : apoptotic cells) for an o.n. Tgf- β mRNA expression is evaluated by qPCR. Each sample is tested in duplicated. Each dot is one sample. Data from two independent experiments are pulled. Mean + SD is reported.

D, F, G, I, L. Col1a1, Tgf-b, Col3a1, Mmp2, Mmp7 mRNA expression is evaluated by qPCR on total liver extracts in vehicle treated mice and at distinct time point after CCl₄ discontinuation. Each mouse is tested in duplicated. Each dot is one mouse. Mean is reported.

E-H. Quantification of COL1A1 and COL3A1 by immunohistochemistry and image analysis. 20x magnification. Min. 20 field/mouse are quantified and averaged. Each dot represents a mouse.

J. a-SMA mRNA expression is evaluated by qPCR on total liver extracts in vehicle treated mice and at distinct time point after CCl₄ discontinuation. Each mouse is tested in duplicated. Each dot is one mouse. Mean + SD is reported.

K. a-SMA immunohistochemistry. 20x magnification. Representative pictures of the liver of Gpnmb⁺ and Gpnmb⁻ mice left untreated or at 48h of recovery after CCl₄ discontinuation.

M. Proliferation in the regenerating liver is evaluated at 48h after the suspension of the CCl₄ using IHC for Ki67. Parenchymal (hepatocytes) cells are distinguished on the basis of the diameter and quantified. 20x magnification. Min. 20 field/mouse are quantified and averaged. Each dot represents a mouse. Mean $\pm$ SD is reported.

N. Representative pictures of the liver of Gpnmb⁺ and Gpnmb⁻ mice left untreated or at 48h of recovery after CCl₄ discontinuation.

A-N. Each dot represents a mouse. Data have been analyzed with two-way ANOVA. Two-way ANOVA is followed by a Bonferroni post-hoc test to compare all pairs of columns. Data have been checked for equal variance before further analysis. *p<0.05

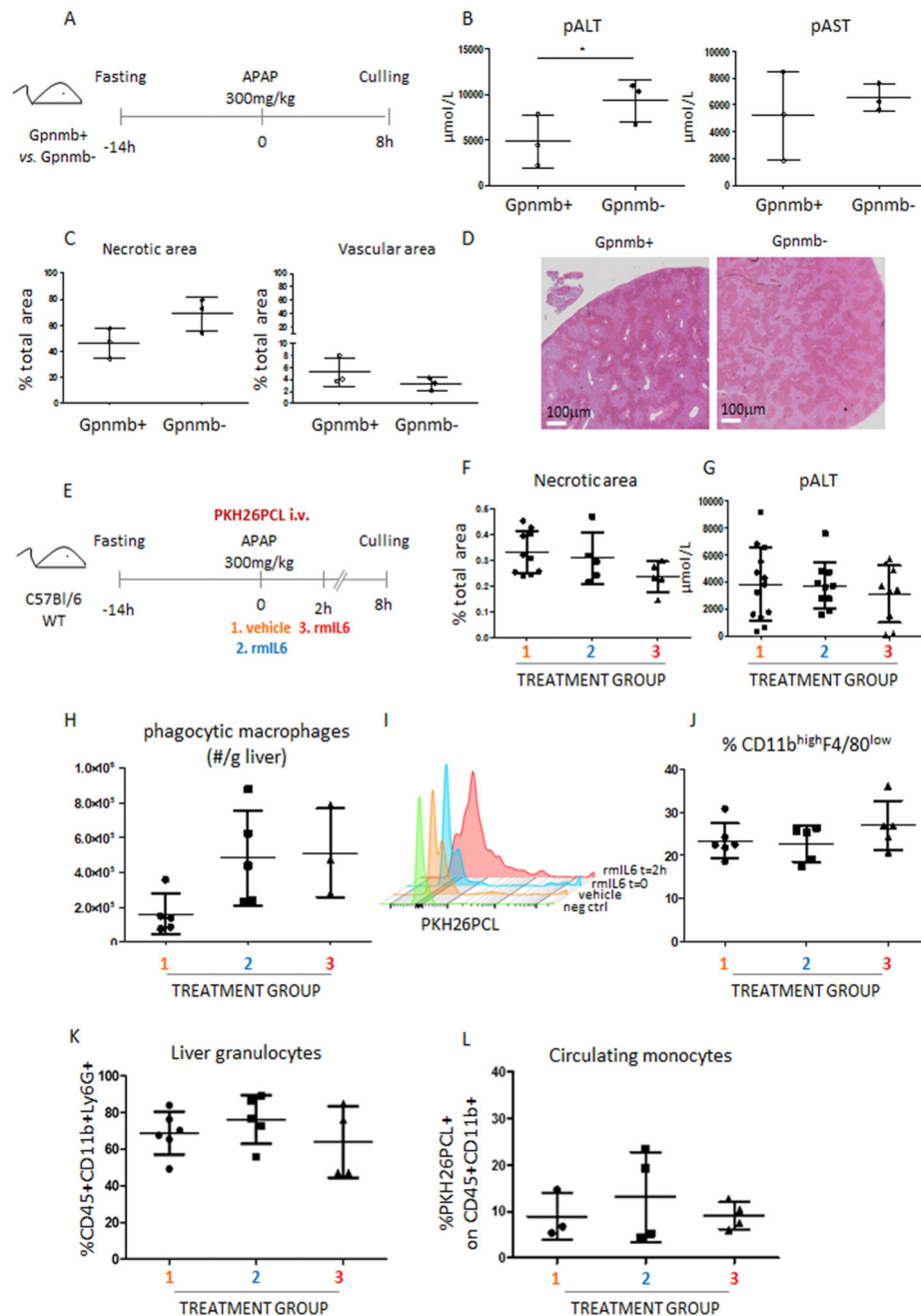


Figure 7. Digestion of the phagocytic cargo limits tissue damage in a model of acute liver injury by acetaminophen overdose, and IL6 treatment improves phagocytosis of infiltrating macrophages

A. Experimental design: Gpnmb⁺ and Gpnmb⁻ mice have been starved for 14h and then dosed with 300mg/kg of APAP and culled 8h after liver damage induction.

B. Plasma (p)ALT and pAST have been measured in Gpnmb⁺ and Gpnmb⁻ mice at cull. Each dot represents a distinct mouse.

C. Image analysis of H&E stained sections. Necrotic area and vascular area is reported in the left panel, vascular area in the right panel.

- D.** Representative pictures Gpnmb+ (left) and Gpnmb- (right) livers at 8h after APAP injection stained with H&E. This is an enlargement of an area of a total-liver picture at 4x magnification. Full-liver picture in Fig.S3E.
- E.** Experimental design: Gpnmb+ and Gpnmb- mice have been starved for 14h and then dosed with 300mg/kg of APAP and culled 8h after liver damage induction. Saline (vehicle, Treatment group 1) or rmIL6 have been injected i.p. at the same time of APAP injection (Treatment group 2) or 2h later (Treatment group 3).
- F.** Image analysis of H&E stained sections. Each dot is a distinct mouse. Mean $\pm$ SD of the necrotic area is reported.
- G.** Average $\pm$ SD of the pALT is reported. Each dot represents a distinct mouse.
- H.** Average $\pm$ SD of the number (#)/g liver of infiltrating phagocytic macrophages (CD45⁺Lin⁻CD11b^{high}, F4/80^{low/int}, PKH26PCL⁺) is reported. Each dot is a distinct mouse.
- I.** Representative histogram plots for PKH26PCL in the gate of infiltrating macrophages in a PKH26PCL uninjected mouse (neg ctrl) and in the three groups of mice analyzed.
- J.** Average $\pm$ SD of the % of infiltrating macrophages (CD11b^{high}, F4/80^{low}) in the gate of CD45⁺Lin⁻ cells is reported. Each dot is a distinct mouse.
- K.** Granulocytes percentage (%) on the parental population is calculated in liver digest of mice dosed with APAP 300mg/kg and injected with saline (Treatment group 1), rmIL6 at the time of APAP injection (Treatment group 2) and 2h after the APAP injection (Treatment group 3).
- L.** % of phagocytic (PKH26PCL⁺) bona fide circulating monocytes (CD45⁺CD11b⁺Ly6G⁻) at the time of culling in the blood of mice dosed with APAP 300mg/kg and injected with saline (Treatment group 1), rmIL6 at the time of APAP injection (Treatment group 2) and 2h after the APAP injection (Treatment group 3).
- B, C, F, G, H, J, K, L.** Data have been analyzed with Student's t-test or two-way ANOVA for unpaired data when appropriate, followed by a Bonferroni post-hoc test to compare all pairs of columns. Data have been checked for equal variance before further analysis.
- *p<0.05 **p<0.01

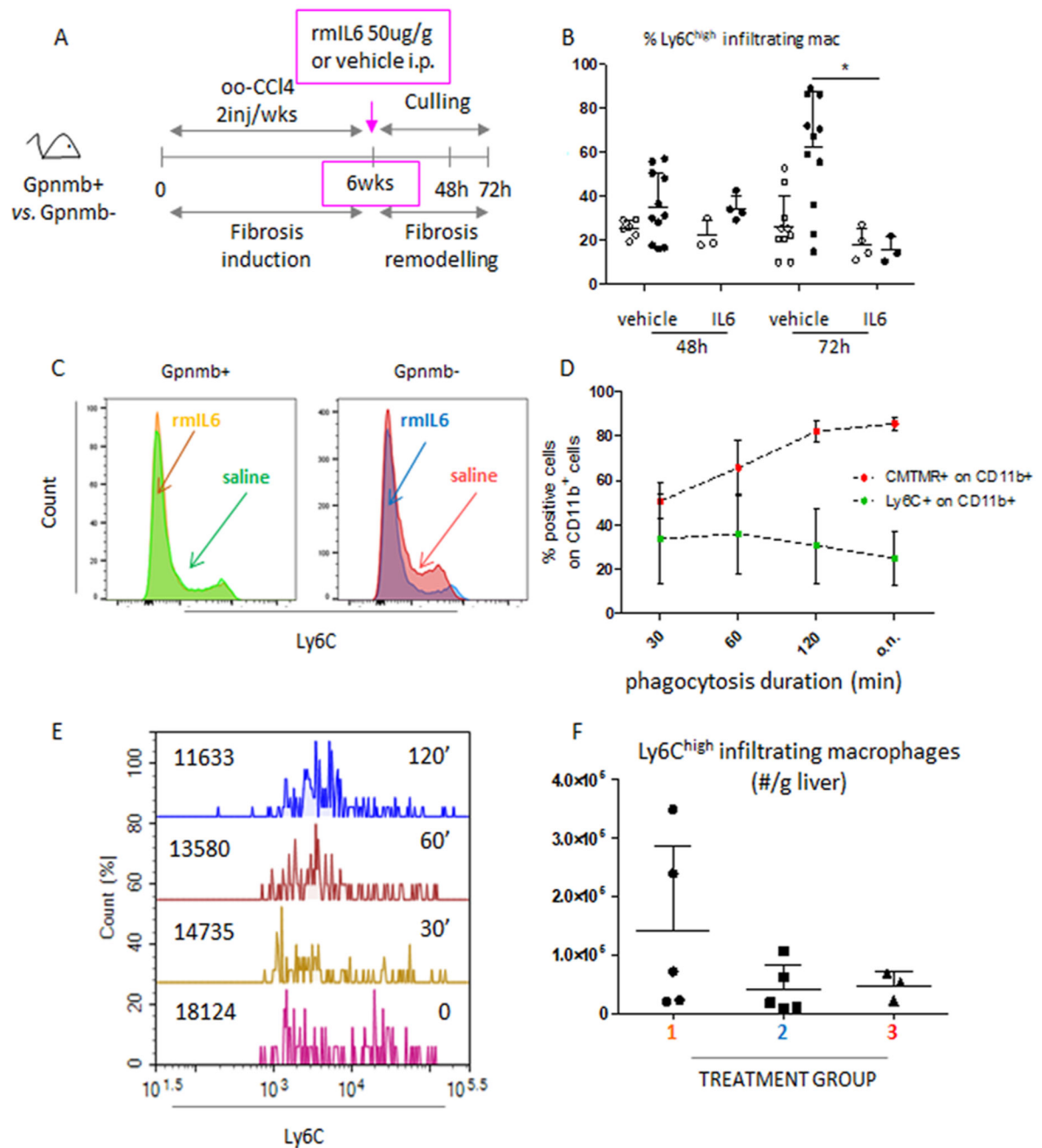


Figure 8. Phagocytosis drives the conversion of inflammatory to restorative macrophages.

A. Experimental design: chronic CCl₄ administration followed by the injection of rmlL6 or vehicle (saline, sham treated mice) i.p. at the suspension of CCl₄. Mice have been culled 48h and 72h later. Livers have been harvested, digested and labelled for flow cytometry.

B. Flow cytometry analysis of liver digest. Infiltrating macrophages have been identified as described in Fig.S3 and inflammatory macrophages are identified as % of Ly6C^{high} events in the gate of infiltrating macrophages. White dots: Gpnmb+ mice. Black dots: Gpnmb- mice. Each dot is a distinct mouse. Average $\pm$ SD are reported. Data have been analyzed with two-

way ANOVA for unpaired data followed by a Bonferroni post-hoc test to compare all columns with the control column.

C. Representative histogram plots of Ly6C fluorescence in the gate of infiltrating macrophages in the liver digest of Gpnmb⁺ and Gpnmb⁻ mice.

D. Green: expression of Ly6C in BMDM subjected to phagocytosis of apoptotic thymocytes at indicated time point. Red: % of phagocytic BMDMs, calculated on the same population analyzed for Ly6C expression. Average $\pm$ SD is reported. n=3

E. Representative histogram plot of Ly6C expression at distinct time points after the start of phagocytosis. We report MFI values on the left and the time point analyzed on the right.

F. #/g liver of Ly6C^{high} infiltrating macrophages in APAP dosed mice untreated (1, orange) or treated with rmIL6 at the moment of APAP injection (2, light blue) or 2h later red, 3. Each dot is a distinct mouse. Average + SD are reported.

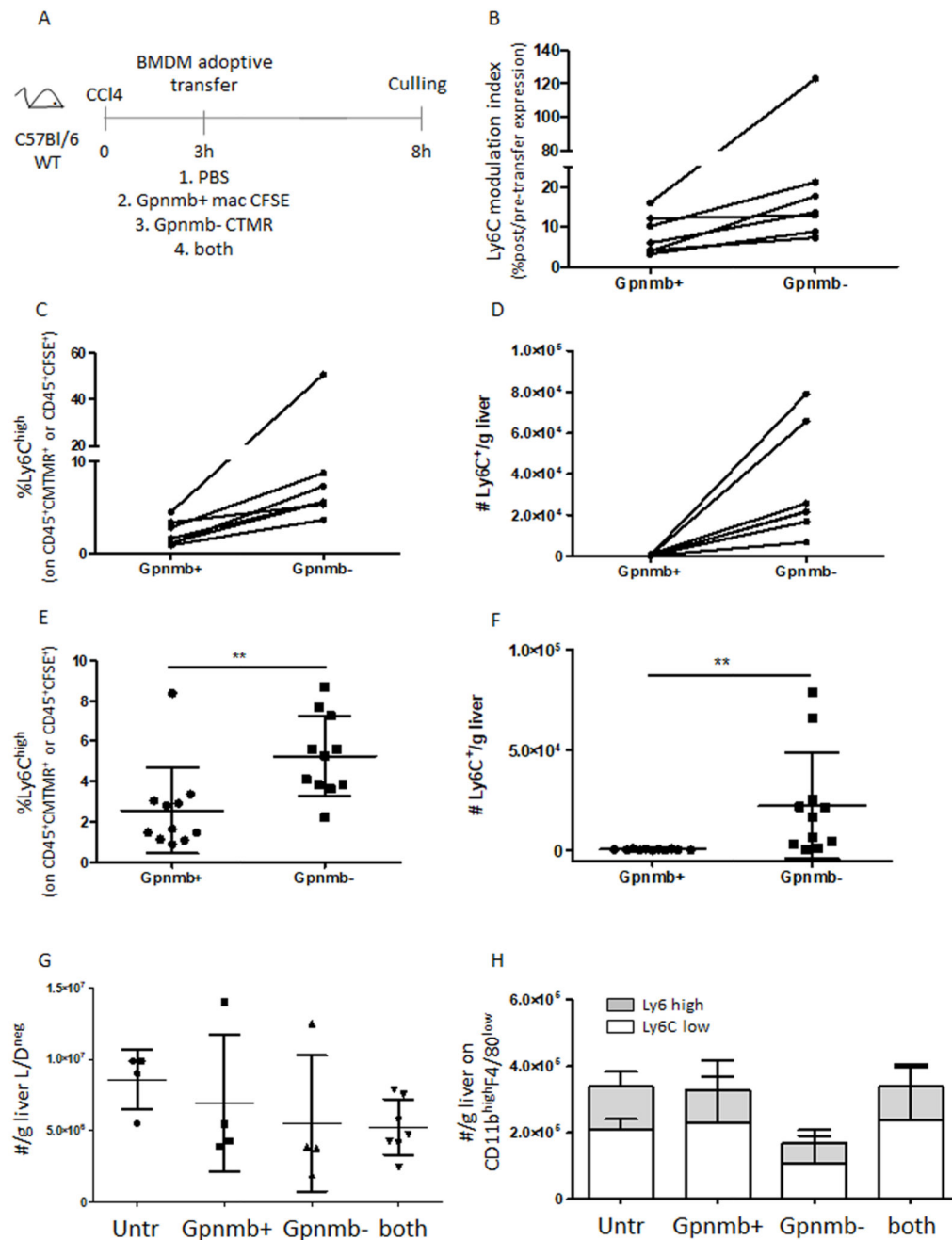


Figure 9. Phagocytosis drives the conversion of inflammatory to restorative macrophages in a cell autonomous manner

A. Experimental design: single CCl₄ injection is followed 3h later by adoptive transfer of BMDMs. Group1: PBS; Group2: Gpnmb+ BMDMs labelled with CFSE; Group3: Gpnmb- BMDMs labelled with CMTR; Group 4: both populations in equal number. The experiment has been repeated with Gpnmb+ CMTMR BMDMs and Gpnmb- CFSE BMDMs. 8h after CCl₄ injection mice have been culled, liver harvested, digested and labelled for flow cytometry analysis.

- B.** Ly6C modulation index = Ly6C expression in the Gpnmb⁺ or Gpnmb⁻ gate/Ly6C expression pre-transplant. Each couple of data represents the level of Ly6C of BMDMs transferred in the same mouse (group 4).
- C.** % Ly6C^{high} in the transplanted population, without considering the levels of Ly6C expression pre-transplant. Each dot on the left is the % of Gpnmb⁺ Ly6C^{high} in one transplanted mouse. Each connected dot on the right is the % of Gpnmb⁻ Ly6C^{high} in the same transplanted mouse.
- D.** Same analysis as in **C.** carried out using the number (#) of Ly6C^{high} transferred BMDMs.
- E.** Analysis of the % Ly6C^{high} events in the gate of Gpnmb⁺ or Gpnmb⁻ adoptively transferred BMDMs, including mice that received only one of the two populations (group2+4 vs. group3+4). Mean and SD are reported. Data are analyzed by t-test for unpaired data after the distribution is checked for equal variance. **p,0.01.
- F.** Same analysis as in **E.** carried out using the number (#) of Ly6C^{high} transferred BMDM.
- G.** The mean and SD of the number (#) of alive cells/g liver is reported. Each dot is a mouse.
- H.** # of Ly6C^{high} and Ly6C^{low} infiltrating (CD11b^{high}-F4/80^{low}) macrophages/g liver in mice receiving PBS (uninjected), Gpnmb⁺ BMDMs only (Gpnmb⁺), Gpnmb⁻ BMDMs only (Gpnmb⁻) or both populations (both).